Effect of chlorhexidine in the treatment of fungal corneal ulcer in Bangladesh perspective.

PhD. Thesis

Submitted by

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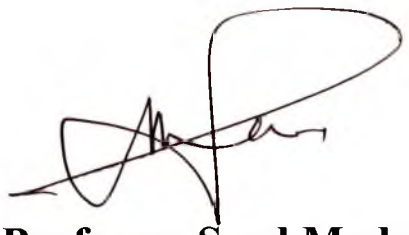
FOR
MY PARENTS
LATE MD. YUSUF MOLLA
&
MST. AYESHA BEGUM

Congratulations for a successful piece of work, and all good wishes for the future.



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DECLARATION

The work presented in this thesis is original and has not been submitted by me to any university or institution for the award of any degree or diploma. The thesis **"Effect of chlorhexidine in the treatment of fungal corneal ulcer in Bangladesh perspective"** is submitted by me for the award of the degree of doctor of philosophy under the university of Dhaka is based on my own work carried under the supervision of Professor Syed Modasser Ali, DO; FRCS; FRCOphth; FCPS.

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Md. Rezanur Rahman

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ABSTRACT

Suppurative corneal ulcer is a common problem in the tropical developing countries. Antibiotics are available but a very few antifungal agents are available there in big cities. In rural areas antifungal agents are not always available there. Microbiological facilities are not available to identify the causal organisms in rural areas of Bangladesh. In addition ignorance, poverty, and illiteracy cause patients to use harmful traditional eye medicines, leading to blindness. In a clinical trial chlorhexidine gluconate 0.2% showed good response against fungal corneal ulcer without any toxicity. In this study, I have explored the use of chlorhexidine gluconate in suppurative corneal ulcer which should be cheap. Chlorhexidine is effective against Gram positive and Gram negative bacteria. Chlorhexidine has already been prescribed for the treatment of Acanthamoeba keratitis. It is also active against *Chlamydia trachomatis*. Chlorhexidine is also cheap, stable in the tropical temperature and has already been used as an antiseptic and as a preservative for more than 40 years. I therefore suggest that chlorhexidine may be a useful first line agent in any suppurative corneal ulcer when microbiological facilities and antifungal agents are not available.

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CHAPTER: I

INTRODUCTION

1.1 CORNEAL BLINDNESS:

Corneal blindness is the second most important cause of blindness in the world after cataract. In contrast to the visual loss from cataract which affects mainly the older age group, corneal ulceration is encountered in all ages. About one-quarter to one-third of the estimated 38 million cases of blindness in the world are due to corneal damage[1].

Trachoma is still the most important cause of corneal blindness and may still account for 6 to 7 million cases of blindness in the world[1]. It is the second common cause of blindness next to cataract in Africa. In Mali it is the prime cause of blindness[11]. In north-west Kenya, trachoma and xerophthalmia are the major causes of blindness up to age of 35 and above the age of 45 it is cataract[9]. In the Northern Transvaal, trachoma is second cause of blindness accounting about 10% and women are mainly affected may be due to lower literacy[10]. However, not all these surveys distinguish between trachoma and other causes of corneal opacity. In those that do, causes other than trachoma may be very important. In Gambia non-trachomatous corneal opacity is more than the trachomatous opacity and accounting about 20% and 17% respectively[7]. In China corneal opacity due to trachoma varies from 2% to 26% while 7% to 30% is due to non-trachomatous corneal opacity[11].

About 30% of all blindness in some developing countries is caused by corneal opacity. It may be caused by a wide variety of species of bacteria or filamentous fungi. Sometimes corneal ulcers are caused by free living acanthamoebas. The most common bacteria are *Streptococcus pneumoniae*, *Pseudomonas aeruginosa, Staphylococcus aureus* and *Staphylococcus epidermidis* and the most common fungi are *Fusarium* and *Aspergillus* species (Appendix-1). *Acanthamoeba polyphaga* keratitis was first identified in a South Texas rancher after corneal trauma in 1973[3], since then 68 cases were reported upto 1988, mostly in the United States[2]. A source of contamination can often be identified and an increasing number of case of Acanthamoeba keratitis have been associated with the wearing of soft contact lenses[5].

Minor trauma to the eye shortly before the onset of the keratitis was documented in most of the published reports. Hay, dust, sawdust, water and the use of contact lenses have been associated with the traumatic event[4]. A population-based survey of the prevalence of major causes of blindness and visual impairment was conducted in Bisha region, Saudi Arabia. Overall, 2882 people were examined and corneal opacity which causes visual impairment is 3.8%. and the causes of corneal opacity is same for trachoma and other infections[13]. In one study in Eye Infirmary and Training Complex (EITC) Chittagong, Bangladesh, showed about 60% of suppurative corneal ulcers were caused by bacteria 40% by fungi [45]. The other cases of corneal blindness in the world are due to corneal damage, Xerophthalmia, Trauma, Neonatal gonorrhoea, Leprosy, Onchocerciasis and harmful medical or non-medical eye practices. Most of the developing countries are in the tropical region. In the tropical countries about 30% of suppurative corneal ulcer is caused by fungus. On the other hand it is very difficult to tell the causative organisms by seeing the appearance of the corneal ulcer. The microbiological facilities are not always available in the developing countries to identify the organisms. There are no facilities to do the antifungal susceptibility tests in the developing countries. It is shown that chlorhexidine gluconate 0.2% is effective against fungal keratitis [80,81,82]. Chlorhexidine has already been prescribed for the treatment of Acanthamoeba keratitis [39]. Chlorhexidine has got wide range of antibacterial action against both gram positive and gram negative bacteria [36].

In the developing countries there is indiscriminate use of drugs, including even steroids as they are easily purchasable from any pharmacy and freely prescribed by village doctors. Antifungal drugs are not available and fungal keratitis is very difficult to treat. In Nepal, in spite of all available treatment the cause of unilateral blindness from corneal ulceration is 7.9% of all blind eyes[8]. Each year more than half of total number of eyes enucleated in the Eye Infirmary & Training Complex (EITC), Chittagong, Bangladesh come from patients with uncontrolled infection[46]. Keratitis due to filamentous fungi tends to occur in agricultural and out door workers. In developing countries agricultural trauma is the important risk factor for fungal keratitis. Most of the affected people live in rural areas where medical facilities are not available. The academic hospitals are situated in big cities and there continues to be a lack of ophthalmic microbiological facilities, ophthalmic trained nursing staff, equipment, beds and other trained personnel. In developing countries there are also logistic problems. Patients come with advanced stages of corneal ulcers so it is usually impossible to differentiate fungal or bacterial corneal ulcer clinically. The cost of microbiological services is very high. Due to lack of medical

facilities, illiteracy, logistic problems and poverty, people are dependent on Ayurvedic medicines, traditional eye medicines, snail juice, rose-water etc. which leads to blindness.

1.2 Hypothesis:

Ocular use of chlorhexidine gluconate 0.2% is an effective alternative for the treatment of suppurative fungal corneal ulcer.

CHAPTER: II

THE AIMS AND OBJECTIVES

General objectives:

1. To prevent the blindness due to suppurative corneal ulcers.

Specific objectives:

1. To identify a single antimicrobial agent which is effective against fungi, bacteria and acanthamoeba.
2. The aim is that in any suppurative corneal ulcer it could be used when microbiological services are not available.

CHAPTER: III

LITERATURE REVIEW

3.1. Previous reports of causes of microbial keratitis

Microbial keratitis results from a complex interaction between a wide array of pathogens and a diversity of host responses. The variability of these factors has hampered the development of simple management guidelines that will ensure an optimal outcome for most cases. The consequence of this has been the use of an extensive range of culture media in investigation, toxic levels of antibiotic treatment and constant clinical review.[38] The studies of fungus in the different countries and latitude show that the spectrum of micro-organism responsible for suppurative corneal ulcer varies according to geographic location (Appendix-1). So the understanding of distribution and causative organisms of corneal ulcer all over the world is essential to prevent blindness from corneal ulcer.

Fungal corneal ulcer in tropical and sub-tropical regions is predominantly caused by filamentous fungi rather than yeast species. In tropical regions environmental factors such as humidity, rain fall, temperature, and wind accounts for fungal keratitis[46,47]. In South Florida the incidence of fungal keratitis increases in the month of November when the climate becomes dry and windy[47]. Wind speed was believed to stimulate frictional or mechanical turbulence, causing eddying and thereby aiding the upward and lateral dispersion of fungal spores. In one study from Florida there was no fungal keratitis in contact lens wearers[51]. The most common organism responsible for fungal keratitis on a world wide basis is *Aspergillus sp*[48]. *Aspergillus* is the important causal organism of fungal keratitis in Bangladesh, Nepal and India. But in Aravind eye hospital, Madurai, India, fungal keratitis is 50% and most important causal organism is *Fusarium* (personal communication; M. Srinivasan). *Fusarium sp.* have been isolated from cornea in cases of fungal keratitis through out the world including north, central and south America, Europe, Africa, Middle east, India, China, Japan and Bangladesh[48]. But *Fusarium sp.* is the most important cause of fungal keratitis in South Florida. In northern United States *Candida sp.* and *Aspergillus sp.* are isolated more frequently in fungal keratitis where as *Fusarium sp.* is the major etiologic agent in the Southern United States[48]. The causal organisms are also changing in the same location and new fungi are isolated. In South Florida

Fusarium solani was the commonly isolated organism between 1959 & 1977, but *Fusarium oxysporium* being the most commonly isolated organism between 1982 & 1992[48]. In South Florida 4 new fungi include *Candida parapsilosis*, *Candida tropicalis*, *Aspergillus terreus*, and *Trichosporon begellii* were isolated from fungal keratitis[48]. In Bangladesh a new fungus *Dichotomophthoropsis nymphaerum* has been isolated[49]. In fact more and more fungus are isolated especially from tropical regions and the number of species of fungus is more in tropical regions than the other parts of the world. It is seen that the highest incidence of fungal keratitis is in Bangladesh and India but the number of causal organisms isolated are more in South Florida and Nepal.

It is also seen that closer to the equator the more the number of species and more away from the equator the less the number of species. In Bangladesh and India more species are yet to be identified. Some of the underlying factors for this large incidence of corneal ulcer in Bangladesh may be due to vitamin and protein deficiencies. The percentage of fungal corneal ulcer is more to the equator than away from equator and all the countries studied here are situated north to the equator except South Africa. As South Africa is 26° south of the equator and at a high altitude the fungal keratitis may be less there. A brief outline of the distribution of corneal ulcer is in Table 1 and Graph 1.

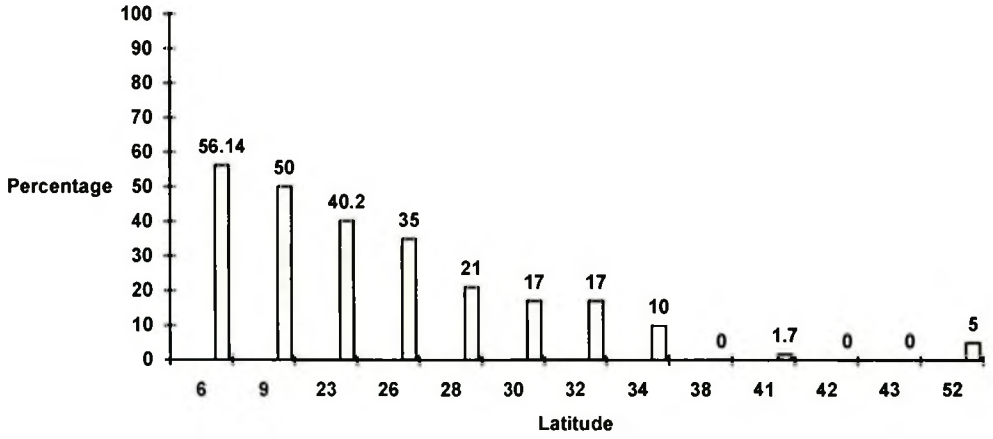
Table no. 1

Geographical distribution of fungi.

Sl.no.	Geographical location	Fungus	Latitude	No. of species.	References
1.	Ghana (Accra)	56.14%	6° (North)	17	41
2.	India (Madurai)	50%	9° (North)	7	14
3.	India (Trichurapalli)	30%	11° (North)	4	54
4.	Bangladesh (Chittagong)	40.2%	23° (North)	14	46
5.	South Florida (Miami)	35%	26° (North)	30	47,12
6.	South Africa (Soweto)	7%	26° (South)	8	56
7.	Nepal (Kathmandu)	21%	28° (North)	34	8
8.	Houston Jones	17%	30° (North)		52
9.	Texas	17%	32° (North)	-	12
10.	California (Los Angeles)	10%	34° (North)		52
11.	San Francisco	0%	38° (North)		52
12.	New York	1.7%	41° (North)	4	12
13.	Boston	0%	42° (North)		52
14	Toronto, (Canada.)	0%	43° (North)		6
15.	London	5%	52° (North)	2	57

Graph-1

Fungi as a percentage of suppurative keratitis in relation to latitude



In the last three decades the number of reported cases of fungal keratitis has increased dramatically possibly due to increased awareness of the disease, advances in laboratory diagnosis and increased use of topical steroids and broad spectrum antibiotics[48]. Corticosteroids are believed to predispose to mycotic keratitis by suppressing ocular immune mechanisms. They also seem to encourage the growth of fungal opportunists. Corticosteroids may activate non-pathogenic fungi and increase virulence of pathogenic fungi[48]. In South Florida each year about 45% cases of suspected infectious keratitis are culture negative due to partially treated bacterial and fungal keratitis[47], otherwise the percentage of fungal keratitis might be higher.

Effective treatment of corneal ulcer depends on early recognition of responsible pathogens. However we do not necessarily require to identify the specific organism. What we need is to differentiate bacteria, fungus and acanthamoeba. Then we need to develop a simple method to culture the organisms and to see the sensitivity against 2 to 3 antifungal agents which could be made available in that country. These methods should be very simple so that local ophthalmologists and general physicians will have practical methods of controlling fungal corneal ulcer in rural areas. At the same time it should be cheap so that every body may be benefited.

3.2. Ophthalmic antibiotics

A. Penicillins.

Mechanisms of action.

They interfere with the biosynthesis of the peptidoglycan structure of bacterial cell walls; cause a decrease of murein hydrolase, which then permits autolysis to occur; and have effects on the intracellular enzyme system[53].

Naturally occurring penicillins

1. Aqueous crystalline penicillin G
2. Procaine penicillin G
3. Benzathine penicillin G

Semisynthetic Penicillins

1. Ampicillin
2. Amoxicillin
3. Hetacillin
4. Carbenicillin Indanyl
5. Methicillin
6. Carbenicillin

Methicillin

It is the preferred semisynthetic penicillin for use against unidentified gram-positive infections. Although naficillin, oxacillin and cloxacillin are more active by weight than methicillin is against penicillinase-producing *staphylococci*, these differences are probably insignificant because of adjusted dosages and degree of serum binding[50]. Protein binding theoretically reduces the amount of free antibiotic available and restricts its distribution in the extravascular space. The in vitro minimal inhibitory concentration of methicillin is not altered in serum, as to an eight fold or greater increase of the other penicillinase-resistant penicillins. It is reasonable to propose that the proteinaceous exudate and the stromal infiltration of corneal ulceration bind antibiotics and thereby reduce the amount of available free agent. All of the semisynthetic penicillins are less effective by weight than penicillin G against

non-penicillinase-producing *Staphylococci*, diplococci and *Streptococci*. Methicillin has the same side effects associated with the other natural and semisynthetic penicillins and may also produce a specific form of nephropathy.

Carbenicillin

It is the first semisynthetic penicillin effective against *Pseudomonas* and *Proteus*. Its bactericidal activity is exerted by inhibition of the cell wall synthesis of sensitive organisms and inducement of the formation of spheroblasts[50]. It is less effective than penicillin G against sensitive gram-positive cocci and is not active against penicillinase-producing *Staphylococci*. It shares the toxic and allergic reactions of the penicillins and may induce haemorrhagic phenomena within the gastrointestinal tract and urinary bladder.

B. Aminoglycosides

Mechanisms of action

This disrupts the cycle of ribosomal function by interfering with the first step of protein synthesis. They also induce misreading of the genetic code of messenger RNA template, incorporating incorrect amino acids into growing polypeptide chains[53].

1. Tobramicin.
2. Streptomycin
3. Neomycin
4. Kanamycin
5. Amikacin
6. Gentamycin

Gentamicin

On the basis of its bactericidal spectrum, stability, intraocular penetration, efficacy and low dose toxicity, gentamicin is the preferred antibiotic for use against unidentified gram-negative infections. Its bactericidal activity is accomplished by inhibition of bacterial protein synthesis. It is effective against 90 percent or more of strains of *Pseudomonas, Aerobacter, Klebsiella, E. coli, Serratia marcescens* and other Enterobacteriaceae[50]. *Moraxella* species are variably sensitive. In vitro it is

active against penicillinase and non-penicillinase-producing *Staphylococci* and has been clinically effective in serious staphylococcal infection. Gentamicin shares some of the toxic effects of the other aminoglycosides and becomes ototoxic if serum levels exceed 10 μg per millilitre.

C. Fluoroquinolones

1. Norfloxacin
2. Ofloxacin
3. Ciprofloxacin

They are less toxic than amynoglycosides and are all available as 0.3% solutions. Clinical trials of these in keratitis are only available for ciprofloxacin 0.3% and have shown similar or better results than for conventional therapy. In vitro antimicrobial sensitivity studies show that norfloxacin is less active against Gram-positive isolates than either of the other two quinolones for non-ocular isolates and this has been confirmed for ocular isolates in comparison with ofloxacin[38]. For these reasons norfloxacin is the least appropriate of these available quinolones as broad spectrum monotherapy for bacterial keratitis. Either ciprofloxacin or ofloxacin should be effective as monotherapy for most cases of bacterial keratitis. Ofloxacin penetrates the cornea better than other commercially available fluoroquinolones and human tear film concentrations of ofloxacin, 4 hours after topical administration, exceed the MIC for a wide range of ocular isolates. Ofloxacin, unlike ciprofloxacin, does not induce corneal plaques. These data suggest that ofloxacin should be as effective as ciprofloxacin for bacterial keratitis.

D. Polypeptides

1. Gramicidins
2. Polymyxin B and E
3. Bacitracin

Bacitracin

Although the penicillins may be applied topically, bacitracin is an effective substitute and may minimise the risk of penicillin sensitisation. This agent achieves its bactericidal activity by binding to cell membranes to produce false pores and flux of ions. It is effective against Gram-positive cocci, including penicillinase-producing *Staphylococci* and *Neisseria*[50]. Allergic sensitisation or primary irritation following topical application is rare. Nephrotoxicity prevents parenteral administration.

E. Cephalosporins

Mechanisms of action

They inhibit a transpeptidase completely, thereby preventing a cross-linkage of new murein sub-units. This action results in a structurally weakened cell wall marked by accumulation of cytoplasm and rupture of the cell wall, caused by increased osmotic pressure[53].

First-generation Cephalosporins

1. Cephalothin
2. Cephaloridine
3. Cephalexin
4. Cefazolin
5. Ceftezole
6. Cephradine

Second-generation cephalosporins

1. Cefamandole
2. Cefoxitin
3. Cefaclor
4. Cefuroxime

Cefuroxime

It is a second generation cephalosporin. The cefuroxime levels in primary aqueous humour of humans, when measured between 30 minutes and 6 hours, are higher after I.V. than I.M. administration[53]. These levels are significantly greater than the MICs of most ocular pathogens. Its poor antibacterial activity against pseudomonas, however, necessitates a combination treatment for severe infections, when no causative organisms can be detected.

Third-generation cephalosporins

1. Cefotaxime
2. Ceftizoxime
3. Cefoperazone
4. Moxalactam
5. Ceftazidime

F. Tetracyclines

They are bacteriostatic and induce inhibition by interfering with the protein synthesis of bacteria. They are the drug of choice for chlamydial infection, including inclusion conjunctivitis, trachoma, lymphogranuloma venereum and psittacosis[53]. They are also the drug of choice for blepharitis and conjunctivitis caused by susceptible organisms. Recently, minocycline has been shown to be effective against experimental ocular toxoplasmosis53.

G. Macrolide and related

1. Erythromycin
2. Clindamycin
3. Lincomycin
4. Vancomycin
5. Chloramphenicol

Chloramphenicol

Chloramphenicol exerts its bacteriostatic activity through the inhibition of protein synthesis. Most Gram-positive and Gram-negative organisms are susceptible, including *N. gonorrhoeae*. It is effective against Mycoplasma, Rickettsiae, Chlamydia and Spirochetes[53]. Topical administration of chloramphenicol 0.5% solution has been shown to achieve effective antibacterial levels in the aqueous and cornea.

H. Sulfonamides and sulfones

The action is bacteriostatic. They act by competing with extracellular para aminobenzoic acid (PABA), a substance that susceptible organisms require to form folic acid. Folic acid is essential for the production of purines and to the ultimate formation of the nucleic acid. They have been used in trachoma, gonococcal ophthalmia, streptococcal membranous keratoconjunctivitis, blepharoconjunctivitis and acute dacryocystitis[53].

3.3. Antifungal drugs

1. Polyenes:

Amphotericin-B- either fungistatic or fungicidal
Nystatin-both fungistatic and fungicidal.
Natamycin (Pimaricin)-fungicidal
Etruscomycin (Tetraene).

2. Azoles:

A.Imidazoles: (Two Nitrogen atoms)

Clotrimazole-fungicidal.
Econazole
Ketoconazole
Miconazole-fungicidal.
Isoconazole
Sulconazole
Tioconazole
Oxiconazole
Thiabendazole

B.Triazoles: (Three Nitrogen atoms)

Fluconazole
Itraconazole
Saperconazole

3.Pyrimidines:

Flucytosine (Fluorinated) or 5-fluorocytosine-fungistatic.

4.Diamidines:-Fungistatic

Propamidine
Dibromopropamidine
Hexamidine
Iodohexamidine
Hydroxystilbamidine isethionate

5. Chlorhexidine:- Fungicidal.

6. Povidone-Iodine:- Fungicidal.

7. Silver sulphadiazine.

8. Aureofuscin

9. Fatty acids and their salts:
   Proprionates
   Undecylenic acid

10. Tolnaftate

11.Haloprogin

12.Benzoic acid and salicylic acid.

13.Miscellaneous:
   Griseofulvin-fungistatic.
   Terbinafine
   Acrisorcin
   Candicidin
   Chlordantoin
   Carbol-fuschin
   Sulphur and Thiosulphates
   Iodoquinol (diiodohydroxyquin)
   Clioquinol (iodochlorohydroxyquin).

The available antifungal drugs reach fungistatic but rarely fungicidal, levels in the tissue. The antifungal drugs which are fungicidal show fungistatic action when applied to the cornea because low concentration of drugs are used on cornea while in high concentration they shows fungicidal property. The objective of antifungal therapy is to inhibit fungal growth over a long period so that the body's defence mechanism can manage the fungus[30]. Fungal infections in industrialised countries are frequently associated with a defect in host resistance which should , if possible, be corrected otherwise drug therapy may fail.

Mode of action:

Polyenes:- (Nystatin, Amphotericin, Natamycin).They combine with the sterol of cell membranes and result in an increase in the permeability of the membranes allowing leakage of small molecules.
Imidazoles:-(miconazole,econazole, clotrimazole), permeate the chitin of the fungal cell wall and increases the membrane permeability to various intracellular substances.

Diamidines:-(propamidine, dibromopropamidine, hexamidine, iodohexamidine)
Inhibit the oxidative metabolism of bacteria.

Antifungal drugs for ocular use are usually not commercially available. The following are used in Moorfields Eye Hospital which is one of the specialist reference centres for the United Kingdom.[32,27]

Routes:

1. Intravitreal
2. Subconjunctival
3. Systemic
4. Topical

Systemic route:

1. Flucytosine
2. Ketoconazole
3. Itraconazole
4. Amphotericin-B (toxic)

Topical route:

1.Amphotericin-1/2 or 1 hour interval and reduce doses as condition improves continue may be month. Normally active against *Candida.*
2. Miconazole-same doses
Active against *Candida* and Gram positive bacteria.

3. Clotrimazole-same doses
Active against *Aspergillus, Candida* and *Acanthamoeba.*
4. Econazole-same dose
Active against *Fusarium, Aspergillus, Penicillium.*
5. Natamycin 5% drop-same dose
Active against *Fusarium, Aspergillus, Candida.*

Amphotericin-B.

No intraocular penetration except by direct intraocular injection. Because of poor penetration, it cures only superficial infections when applied topically[30]. It is reported that 0.15% solution of amphotericin B is non-toxic and non-irritant to the eye[54]. Intravenous injection should start with 250 microgram/kg daily, gradually increasing the dose if tolerated to 1 mg/kg daily, maximum 1.5 mg/kg daily or on alternate days[27]. Amphotericin-B is not absorbed from the gut and is the only polyene antibiotic which can be given parenterally[27]. The antibiotic is either fungistatic or fungicidal, depending on the concentration of the drug and the sensitivity of the fungus and it is without effect on bacteria, rickettsiae or viruses[15].

Nystatin

It is not absorbed from the gut but is too toxic for parentral use[27]. Nystatin is reasonably well tolerated in the eye as a 3.3% ointment. It has a medium level of activity against most *Candida* isolates and an occasional isolate of the other ocular fungal pathogens and is active against superficial infections[30].

Natamycin.

A 5% ophthalmic preparation is commercially available. It has got a wide range of antifungal activity against ocular pathogens. It is the least irritating and least toxic of the Polyene antifungals[30]. It can be used as a first line of treatment in any suspected fungal infection. It is relatively stable and can be sterilised by heat without loss of potency. It can certainly cure many superficial fungal infections but is ineffective against deeper infections in the cornea[30].

Clotrimazole

It has got a broad spectrum antifungal activity with low human toxicity. Topically it is as good as the Polyenes. In severe infections it is used both topical and oral administration of 60 mg/Kg/day to 100 mg/Kg/day. In children 150 mg/kg/day[30]. It can be given to patients with renal dysfunction induced by Amphotericin-B but should not be given in the first three month of pregnancy and to those with severe liver or adrenal disease[30]. It is active against *Aspergillus sp*[28]. infection. Clotrimazole 1% solution in arachis oil is recommended[32].

Miconazole.

This is a broad spectrum antifungal with low mammalian toxicity[30]. It is as active as Benzylpenicillin against Gram positive bacilli and cocci. It is not significantly absorbed when applied topically but is partially absorbed at oral administration. A daily oral dose of 0.3 Gram is well tolerated. It does not induce drug metabolising enzymes in liver. In severe infections it can be given intravenously. Topical 1% solution is as effective as Nystatin. It is active against *Candida sp*[30].Topical use of this compound is sometimes associated with superficial punctate keratitis[34].

Econazole.

This is a broad spectrum antifungal, which does not induce drug-metabolising enzymes in the liver. The oral dose is 0.3 gram/day. Topical 1% solution is active against *Fusarium sp., Aspergillus sp.* and *Penicillium*[30]. It is less irritating and can be given intravenously in severe infection. Jones and associates considered econazole to be most widely acting drug for the treatment of mycotic keratitis when compared to other azoles[34].

Thiabendazole

It is an antihelmintic. It penetrates the eye well in a 4% suspension and is non-irritating to the eye. It is particularly active against some ocular isolates of *Fusarium* species and other filamentous fungi including *Penicillium, Phialophora* and *Cladosporium* species[30]. It can be given orally to back up intraocular penetration from topical administration.

Ketoconazole

The dose is 600mg/day orally and topical 1% suspension. Oral administration may produce effective drug levels in the cornea and aqueous[31]. Combined topical and oral ketoconazole was very effective in treating mycotic keratitis but was ineffective in *Aspergillus fumigatus* keratitis with intraocular invasion. Oral therapy may be associated with a transient rise in certain serum enzymes and topical therapy may result in transient superficial punctate keratitis[34].

Itraconazole

Oral therapy brought excellent responsiveness with mycotic keratitis. It is the drug of choice for treating keratitis due to *Aspergillus spp*[34]. Oral as well as topical therapy could be administered with minimal or no side effects. 1% suspension for topical use has been prepared and is generally well tolerated.

Saperconazole

A triazole derivative. It exhibits broad spectrum activity against many fungi, especially *Aspergillus sp.* and certain dematiaceous fungi and was found active following topical, oral and parentral administration[34].

Fluconazole

A triazole derivative. It penetrates rabbit eyes better than ketoconazole and itraconazole. Topical therapy was found to be useful for keratitis due to *Candida sp*[34]. Oral fluconazole produces effective drug levels in the cornea, aqueous, vitreous and choroid/retina in animals[31].

Flucytosine.

It is well absorbed orally and is remarkably non-toxic in human beings because it is not metabolised but excreted unchanged in urine[30]. It has got a synergistic effect on *Candida* with Amphotericin-B. Oral dose is 200mg/kg/day. Topical 1.5% drops are non-irritating to the eye. Sub-conjunctival injection is disappointing.

Aureofuscin

This is an antifungal agent from China which was used topically as a 0.1% solution or a 1% ointment to treat mycotic keratitis, and a success of more than 80% was reported[34].

3.4. Non-specific antiseptics

Propamidine (Brolene)

Gram positive species are all more susceptible than Gram negative organisms[25]. The unionised propamidine penetrates bacterial cell wall[26]. Dibromopropamidine and iodohexamidine were found to be the most effective of the compounds[25]. It is effective in persistent angular conjunctivitis caused by *Morax-Axenfeld bacillus*[24]. Fungistatic activity was determined by noting the highest dilution which caused complete inhibition of growth of fungus after five days at room temperature[21].

Chlorhexidine.

It is used as topical antiseptic for application to skin, wounds and mucous membrane and for dental use. It has been used as pharmaceutical preservative, particularly ophthalmic solutions and as a disinfectant for items such as inanimate surfaces and instruments[16]. It disrupts the plasma membrane of the bacterial cell. It is poorly absorbed from the gastrointestinal tract and negligibly absorbed from the skin of adults.

Antimicrobial action: The antimicrobial activity is directed mainly toward vegetative gram-positive and gram-negative bacteria. It is inactive against bacterial spores except at elevated temperatures. At low concentrations it is bacteriostatic whereas at high concentration it is bacteriocidal[16]. The in vitro bactericidal and fungicidal activity of 0.05% chlorhexidine gluconate was determined using a procedure based on British Standard 3286 (1960)[16]. Acid-fast bacilli are inhibited but not killed[36]. It is active against some lipophilic viruses e.g. influenza virus, herpes virus, HIV. Fungicidal activity in general is subject to species variation. It also has an effect against *chlamydia*[23]. In high and low concentrations it effectively kills cysts of *Acanthamoeba* spp.[36,79]

Wound healing: It slightly delays wound healing but using in infected wound it actually accelerates the rate of healing.

Skin irritation and sensitisation: It is not skin irritant with any concentration.

Oncogenicity: It is not a carcinogen.

Effect of pH: For antimicrobial activity the optimum range is 5.5 to 7.0.

Storage: Chlorhexidine solution may be stored at room temperature for at least 1 year.

Sterilisation: A solution of less than 1% of chlorhexidine may be autoclaved at 115^0C for 30 minutes or for 15 minutes at 121^0C to 123^0C. A concentration of more than 1% causes insoluble residue.

Solubility: Chlorhexidine digluconate is very soluble but chlorhexidine diacetate is soluble at 1.9% W/V.

Toxicity

A. Cornea.

Animals:

1. Aqueous solutions containing 2%, 1%, and 0.1% chlorhexidine were given one drop in rabbit eyes twice daily for one to seven days. Daily examination of the rabbit eyes receiving the chlorhexidine solutions revealed no gross or microscopical changes. Rabbit corneas treated with a 2% solution of chlorhexidine, two drops four times a day for one week, revealed no changes. Except for its effect of binding mucous to the surface of the lenses, chlorhexidine in the concentrations used, is an ideal bacteriostat. It has the following properties: I. chemical compatibility with ophthalmic drugs, II. wide bacteriostatic and bactericidal activity against those organisms likely to be encountered in both the compounding and use of the solution, III. solubility in water and buffer solutions, IV. no effect on the pH and tonicity, V. non-toxic and non-irritant to the ocular tissues, VI. stable after prolonged storage, VII. heat-stable (permitting autoclaving)[35].

2. In the subacute direct instillation studies in rabbits , a slight circumcorneal injection and conjunctivitis were observed in both experimental and control eyes. A concentration-dependent increase in the incidence of these reactions was noted as the chlorhexidine digluconate content increased from 0.005% to 0.05%. No deleterious ocular responses were observed throughout the gel lens wearing studies. These studies suggest that chlorhexidine digluconate formulated properly has merit as a gel lens sterilising agent[43].

3. Following direct topical application of up to 2% chlorhexidine in rabbits eye, no changes to the cornea were seen by direct observation or light microscopy; however, superficial epithelial changes were noted by electron microscopy following application of 0.1% and 0.5% chlorhexidine[16].

4. Concentrations of greater than 2% were clearly toxic to both the corneal epithelium and conjunctiva: a concentration of 1% produced no significant delay in epithelial healing but did cause mild conjunctivitis. Concentrations less than 1% were not statistically different from the control group either in re-epithelialisation or visible toxic effects[16].

5. Irrigation of rabbits cornea with 2% and 4% chlorhexidine significantly slowed the healing rate compared with saline control. Irrigant concentrations of $\leq$ 1% did not statistically delay healing. Topical aqueous chlorhexidine may be an alternate agent for preoperative cojunctival antisepsis[59].

6. Hibiclens (chlorhexidine 4% and detergent) exposed to rabbit eyes for varying time intervals ranging from 5 to 15 minutes causes severe, irreversible and progressive corneal damage[60].The effect of the detergent was not distinguished from that due to the chlorhexidine.

Human

Occasionally chlorhexidine may cause irritation of corneal epithelium, although it may depend on the formulation used[61].

B. Nervous system

Animals

1. Chlorhexidine caused a marked and dose-dependent degeneration of adrenergic nerves when injected into the anterior chamber of albino rat eye. Two days after the injection of lowest dose of 0.05% chlorhexidine, approximately 30% of the nerves had disappeared. Almost complete degeneration was observed after the same time with higher doses of 0.5%, 1% and 1.5% chlorhexidine. Two weeks after the lowest dose, the nerves had regenerated almost completely. With the highest dose used, only some 40% of the normal adrenergic nerve plexus had reformed after 51 days. This suggest that neurotoxic actions on thin unmyelinated fibre systems should be looked to also in the central nervous system[62].

2. Dose-dependent inhibitory effect of chlorhexidine ($2.5x10^{-5}$-$5.0x10^{-4}$) g ml. on neuromuscular transmission were localised by tension and electromyogram recording during indirect stimulation on the isolated rat phrenic nerve-diaphragm preparation[63].

Human

1. Chlorhexidine has been widely used in medical practice since early 1950s. This extensive experience has demonstrated the virtual absence of sensitisation and a low irritancy potential for the compound. Only one significant adverse effect has been identified during medical use. This is sensorineural deafness after direct instillation of commonly used chlorhexidine into the middle ear cavity[64].
2. Chlorhexidine 1% gel does not affect the adrenergic innervation of buccal mucosa[65].

C. Oral mucosa

Animal

1. There were no statistically significant differences in enzyme activity between the placebo group and individuals who used 0.2% chlorhexidine for 18 months[77].
2. Chlorhexidine (Hibitane) is poorly absorbed after oral administration, and its percutaneous absorption is absolutely minimal. No clinical or histological effects have been obtained in any animal study to cause hesitation in the light of proliferating applications of chlorhexidine in human use[66].
3. Chlorhexidine cause bone loss during the treatment of experimentally induced active periodontitis in dogs[67].

Human

1. Chlorhexidine treatment reduces plaque and gingivitis, but tended to stain teeth when used as 10 ml. of 0.2% aqueous solution of chlorhexidine gluconate daily in addition to tooth brushing and interdental cleansing. Stains were readily removed by a conventional dental prophylaxis procedure. There were no other local side effects relative to the structure and function of the oral mucosa, tongue, salivary glands and pharyngeal complex following the prolonged use of chlorhexidine[68].
2. There was no changes in the normal structure of keratinizing oral epithelia as a result of prolonged daily exposure to 0.2% chlorhexidine for one year[72].
3. During two years of daily use of chlorhexidine mouth rinses, no systemic or local side effects were observed. Chlorhexidine is poorly absorbed following oral dosing and that the major excretory route is in faeces[73].

4. Very occasionally a reversible swelling of the parotid glands has been reported after use of chlorhexidine in mouth rinse formulations. Occasional oral intolerance of mouth rinse formulations has been reported, although no histological abnormalities were present in gingival biopsies taken after 18 months' daily use[64].
5. After periodontal flap surgery 1% chlorhexidine gel effectively reduces pain and swelling compared to penicillin. Chlorhexidine may be a suitable alternative to penicillin after periodontal flap surgery[74].
6. Allergy is extremely rare. Occasionally there may be parotitis or sore mouth. The most common problems are staining of the tongue and the teeth, interference with taste and an unpleasant taste of the compound as prepared. All these effects are rapidly reversible[61].
7. Intensive treatment with chlorhexidine gel, in individually fitted custom trays, combined with meticulous oral hygiene measures may induce toxic effects on the surface layers of the gingiva and mucosa[70].
8. Skin and oral tissue cells in culture were exposed for an hour to 0.005% and 0.002% chlorhexidine and for 30 seconds to 0.12% chlorhexidine. 0.002% showed minimal cytotoxicity but is able to suppress cell division almost completely. Chlorhexidine is highly cytotoxic to cells in vitro, but various cell functions such as proliferation, collagen gel contraction and protein synthesis are affected to different degrees by the drug[69].
9. In Norway, chlorhexidine has been dispensed for over 20 years. But the only side effects were staining of teeth and tongue and dryness of mouth, and occasionally oral ulcerations. Only 4% of the dentists recommended mouth rinse with 0.1% chlorhexidine, whereas 96% recommended 0.2% chlorhexidine[71].

D. Other systems

Animals

1. The effect of 0.05% chlorhexidine in chronic ulcer healing did not differ from saline[75].
2. Chlorhexidine was used as wound irrigation fluids upon femoral arteries and veins in the rats by microsurgical techniques. Chlorhexidine at 0.05%, 0.02% and 0.01% was found by contrast to have a very low toxicity which was comparable to physiological saline[76].
3. 2 ml. of 0.05% chlorhexidine was injected intraperitoneally in rats, 5 minutes after the inoculation of pure *Escherichia coli*. The percentage of neutrophils was superior to the control group[78].

Human

1. Absorption after oral ingestion is very low and long term oral use has not produced changes in haematological and biochemical parameters[64].
2. People who are bathing every day of their lives for years with chlorhexidine have no ill effects[61].
3. While 0.5% chlohexidine is irritant to the bladder of some people, 0.1% is not[61].

Povidone Iodine

It is an effective broad-spectrum disinfectant with no reported toxicity to cornea and conjunctiva when applied topically in single dose to ocular surface[18]. Gram negative organisms are particularly susceptible[20]. Povidone iodine is effective against *Neisseria gonorrhoea, Chlamydia trachomatis*, *Herpes simplex* virus type-II with three different concentrations (5%, 1% and 0.1%)[18]. It is Germicidal in action. It has a broad-spectrum bactericidal activity and is also effective against fungi. As the incidence of fungal infection of the cornea increases with the use of antibiotics and is more dangerous in presence of steroids, if these drugs are to be used for prolonged periods, topical administration of 1% povidone-Iodine reduces the danger of superimposed mycotic infection[17]. It significantly reduces the duration of contamination by the fungus. Povidone-Iodine 5% solution is effective in reducing bacterial recovery from the perilimbal conjunctiva, where most incisions for intraocular surgery occur[19].

Polyhexamethylene biguanide (PHMB)

It is used as an environmental disinfectant under trade names such as cosmocil CQ, Vantocil IB, and Baquacil, varying in purity. Baquacil is available as a swimming pool disinfectant in the USA; it is an effective sanitiser and is algistatic and amoebicidal[39]. Cosmocil CQ is used for the preservation of cosmetics and pharmaceuticals, and PHMB is in some soft-contact-lens disinfectant solutions. PHMB is not manufactured or licensed for therapeutic use. It is generally less effective than chlorhexidine invitro against Acanthamoeba cysts39·

Silver sulphadiazine (SSZ)

It is a substitute for silver nitrate and does not cause argyrial stain. It has a broad spectrum coverage against bacteria, fungus & viruses[33]. SSZ 1% eye ointment 5 times a day for 2-3 weeks has been recommended. Silver sulphadiazine combines the oligodynamic action of silver with the antibacterial effect of sulphadiazine. It is observed that sulphonamide antagonist para aminobenzoic acid (PABA) did not nullify silver sulphadiazine action and that the silver moiety combined in vitro with both DNA and cell membrane[33]. Advantages were thought to be the absence of side effects, economy and it's efficacy in deeper and extensive lesions[29]. It inhibits the replication of pathogens. 1% silver sulphadiazine eye drops are also available[33]. In practice, this preparation is no longer being used in India.

3.5. Antiacanthamoebal drugs

1.Diamidines:-

Propamidine
Dibromopropamidine
Hexamidine
Iodohexamidine

Propamidine (Brolene)

Mr. Peter Wright and Dr. David Warhurst recorded the first medical cure of acanthamoebal keratitis by employing only the topical administration of dibromopropamidine ointment (0.15%), propamidine isethionate eye drops (0.1%), and neomycin eye drops. The minimal inhibitory concentration (MIC) and the minimal amoebicidal concentration (MAC) for propamidine were each less than 1.25 μg per millilitre, and for dibromopropamidine the concentrations were less than 1.25 μg per millilitre (MIC) and 2.5 μg per millilitre (MAC), respectively[4]. They are analoges of stilbamidine and had previously known antibacterial and antifungal effects[4].

Chlorhexidine

In high concentrations it effectively kills cysts of Acanthamoeba sp[36]. 0.02% chlorhexidine digluconate drops in 0.9% physiological saline in combination with 0.1% propamidine isethionate (Brolene) drops, given hourly by day and night for the first 3 days, then 2 hourly by day for 4 weeks, 3 hourly by day for 4 weeks and 4 hourly by day for 4 months had already been recommended for the treatment of Acanthamoeba keratitis[39].

Although compounds such as hydroxystilbamidine, paromomycin, neomycin and miconazole have been found to have variable activity in vitro there has been no published report of medical cure[4]. The disease may be arrested with the use of orally administered ketoconazole and flucytosine, and topical clotrimazole ointment. The disease was arrested after penetrating keratoplasty, orally administered ketoconazole, miconazole eye drops and cryotherapy.[4] *Acanthamoeba* is said to be sensitive to natamycin that disrupts the cell membrane by binding with ergosterol[53]. Polymyxin B produces a disorientation of lipoproteins in the cell membrane, permitting excess permeability[53]. Acridine dye is believed to bind to mitochondrial DNA where it inhibits the synthesis of proteins essential for cellular respiration[53].

3.6. Fungal Culture and sensitivity testing.

Ideally all antimicrobial agents should be discontinued 24 to 48 hours before taking corneal scrapings[31]. The Kimura platinum spatula is ideal but a 21 gauge needle may be used for corneal scrapings. Preservative free proparacaine hydrochloride (0.5%) is less antiseptic than other topical anaesthetics. It is best to use more than one culture media, preferably both solid and liquid, and incubate these media at 37° C and 25°C to 30° C. According to Liesegang and Forster fungal growth usually occurs within 48 to 72 hours after inoculation but O'Day et al recommend that the culture media should be kept for 6 to 8 weeks[54]. Solid media are inoculated by lightly streaking the spatula or loop over the agar surface in rows of C-streaks. Growth on the C-streaks is considered to be significant, growth away from C-streak is considered to be contaminated. Liquid media are inoculated by twirling the loop or spatula in the broth several times[54]. Sabouraud's dextrose agar with gentamicin (50 microgram/ml) is the primary isolation medium for fungi. The medium is nutritionally deficient but the low pH favours fungal growth.[31] Chloramphenicol or penicillin plus streptomycin may be incorporated in to the Sabouraud's dextrose agar medium to suppress bacterial growth[54].

Sabouraud's dextrose agar media (SDA).

Preparation: 65 gm. suspended in 1 litre of distilled water and boil to dissolve completely. Sterilise by autoclaving at 121°C for 15 minute. Antibiotics are used to suppress bacterial growth[22].

Used with drugs:

Before autoclaving: 0.4 gram. chloramphenicol, 0.5 Gram cycloheximide are added to each litre of reconstituted medium.

After autoclaving: 0.5 gram cycloheximide, 20,000 unit penicillin and 40,000 unit streptomycin are added to each litre of autoclaved cooled medium. Chloramphenicol is used to minimise bacterial contamination and cycloheximide to reduce contamination with saprophyte fungi. Cycloheximide can not be used in all media as some pathogens for example *Aspergillus, Histoplasma, Hendersonula, Cryptococcus, Candida* are inhibited by it[22].

Brain-heart infusion broth with gentamicin enhances the isolation of filamentous fungi and yeast[31]. Blood and chocolate agar media support the growth of the majority of fungi at 35°C to 37°C under increased carbon dioxide[31]. Thioglycollate or thiol broth is a semisolid medium that provides adequate redox potentials for the growth of aerobic fungi as well as aerotolerant anaerobes, microaerophillic bacteria, facultative anaerobes and aerobic bacteria[31].

To do antifungal sensitivity tests first prepare the drug solutions, media and inoculum.

1. Preparation of stock drug solution

To prepare a drug stock solution (1280 mg/litre), 64 mg of the drug is dissolved in 50 ml of distilled water[42]. This solution should be filter sterilised and can be stored in small amounts at -20°C for up to 12 months.

2. Preparation of media.

Broth or agar, according to fungus to be tested e.g. yeast or filamentous fungi. Can be obtained from Difco or Oxoid company.

3. Control isolates

Can be obtained from the Mycological Reference Laboratory, Central Public Health Laboratory, 61 Colindale Avenue, London NW9, UK[42].

4. Preparation of inoculum

The yeast inoculum should be prepared from overnight (18 hours) to 48 hours old cultures on Yeast Morphology Agar. 2 ml amounts of sterile distilled water are inoculated of with a loopful of each isolate and the concentration of the suspension adjusted to about 1 x 10^6 cells/ml[42]. When filamentous fungi are being tested, the inoculum should be obtained from 2 to 5 day cultures on Sabouraud's glucose agar. Fungal suspension is prepared by immersing the surface growth in sterile distilled water containing 0.05% Tween 80 and scraping off spores and hyphae with a sterile bent glass rod or wire loop. A spectrophotometer is set at 530 nm to adjust the suspension to 90% transmittance (T). This should result in a concentration of about 1 x 10^6 colony forming units (CFU)/ml. To achieve this density of fungus, it may be necessary to concentrate the original suspension by centrifugation[42].

1. Broth dilution method

In broth dilution tests serial dilutions of the drug are prepared in a fluid medium and then inoculated with a suspension of the fungus under investigation. The drug stock solution (1280 mg/litre) is ten-fold diluted by adding 1 ml to 9 ml of broth[42]. The final concentration is 128 mg/ litre. 11 sterile capped tubes (110 x 16 mm) are placed in a rack and numbered 1 to 11. 1 ml of broth is added to each tube. Then 1 ml of 128 mg/ litre drug solution is added to tube-1 and mixed. Then 1 ml of solution from tube-1 is transferred to tube-2. This serial dilution is repeated through tube-9 and 1 ml from tube-9 is discarded. Then 50 microlitre of the standardised inoculum is added to tube 1 to tube-9 and incubated. Tube-10 is the medium control and tube-11 is the inoculum control. Drug dilution in tube-1 is 64 mg/litre and in tube-9 it is 0.25 mg/ litre.

The MIC is the lowest drug concentration at which there is no visible fungal growth after 48 hours incubation. Growth must be present in the medium control and absent in the inoculum control[42].

2. Agar dilution method

2 ml of concentrated agar solution is added to each of 10 sterile universal container and numbered 1 to 10. 2 ml of the 1280 mg/litre stock drug stock solution is added to container-1, mixed well and transferred 2 ml to container-2 and repeated through container-9. From container-9, two ml is discarded. Melted 200 ml agar is dispensed 18 ml each in 10 sterile universal container placed in 56°C water bath and numbered 1 to 10. Now 2 ml of the corresponding drug containing solutions added to each container of molten, cooled agar, mixed well and poured the contents in to a 9 cm diameter petridishes and numbered 1 to 10. Once the medium is solidified and dried, with a micropipette 20 microlitre of standardised suspension is inoculated and incubated for 48 hours. Plate-10 is medium control and drug dilution in plate-1 is 64 mg/litre and in plate-9 it is 0.25 mg/litre.

MIC is the lowest drug concentration at which there is no visible fungal growth after 48 hours incubation. Growth must be present in the medium control[42].

3. Bioassay

Drug standards are prepared according to the standard method. Media are prepared according to the organisms to be tested. The inoculum is prepared by harvesting the spores from the culture in sterile distilled water and adjusting the concentration to about 1 x 10^6 spores/ml[42]. 1 ml of the spore suspension is added to the melted media and poured into a 25 cm sq. plate and dried. 30 wells of 4 mm diameter are cut in the media and 15 microlitre of standard specimen or patient serum is placed in each well. Each standard and specimen should be tested in triplicate and should be placed on the plate according to the randomised distribution. The plate is incubated for 24 hours. Then the zones of inhibition around each well is measured. The results are analysed by a microcomputer program.

Most antifungals do not diffuse well and give poor or misleading results even when sensitive strains are tested against high drug concentrations. The lowest concentration of the drug to inhibit growth after incubation is referred to as the minimum inhibitory concentration. The minimum fungicidal concentration is defined as the lowest concentration of drug from which sub-cultures were negative. The decision whether an infection with a given fungal strain should be treated with a particular drug, or whether that strain should be regarded as resistant to that drug, is often difficult. It is not unusual to obtain MICs for responsive strains that are much higher than the levels of drug that can be attained in the patient. The MIC obtained often depends on the conditions of the test, with the concentration of the fungal inoculum, the composition and pH of the medium, and the temperature and length of incubation all having a marked effect on the result[42]. The results of MIC determinations must be interpreted with caution because their correlation with clinical effectiveness is often uncertain[42].

CHAPTER: IV

SAMPLE SIZE

Since the confidence interval for universe proportion, $\hat{p}$ is given

$$p \pm z \sqrt{\frac{pq}{n}}$$

Where p = target proportion of fungal corneal ulcer. In this case it is assumed 20% that is

p = 0.2

q = 1- p,

=1- 0.2 =0.80, so

q = 0.80

z = the value of the standard variate at a given confidence level, z is assumed 95% confidence level.

z = 1.96 (From table)

n = sample size.

Then with the given precision rate, the acceptable error e can be expressed as

e = 0.08, (8% assumed).

$$e = z \sqrt{\frac{pq}{n}}$$

$$\text{or } e^2 = z^2 \frac{pq}{n}$$

$$\text{or } n = z^2 \frac{pq}{e^2}$$

$$= \frac{(1.96)^2 \times .20 \times .80}{(.08)^2}$$

$$= \frac{3.84 \times .20 \times .80}{.0064}$$

$$= 96$$

Total number of patients will be 96, half of which will be control group and half of which will be trial group.

CHAPTER: V

METHODOLOGY

Patients attending the out patient department of the National Istitute of Ophthalmology, (NIO) Sher-e-bangla Nagar, Dhaka-1207, Bangladesh, with suppurative keratitis were recruited for the study. Patients with only one eye, diabetes mellitus, already perforated eye, unwilling to participate in the study, pregnant woman and children under1year age were excluded from the study. A double masked randomised clinical trial of 0.2% chlorhexidine gluconate in a total of 96 patient, compared with standard treatment with natamycin 5% was done. The ulcers were scraped for Gram stain, 10% KOH (potassium hydroxide) test and culture. The presence of fungus (hyphal fragments) in Gram stain or in 10% KOH test was taken as the criteria for including the patient in the study. There were two arms of the study, 0.2% chlorhexidine in arm A and 5% natamycin in the arm B. The randomisation was computer generated and the codes A for chlorhexidine and B for natamycin were scaled in the envelopes. The envelopes were numbered serially from 1 to 96. After recruitment of the patients the envelopes were opened serially. The Moorfield Eye Hospital, London supplied 20% chlorhexidine gluconate solution and was diluted with water in the National Istitute of Ophthalmology, (NIO) Sher-e-bangla Nagar, Dhaka-1207, Bangladesh, to prepare 0.2% solution and 5% natamycin was available in the local pharmacy. The NIO authority prepared the solution, labelled the bottles and were stored in refrigerator at 4°C. Corneal srappings were cultured in the in the SDA, chocolate agar media and blood agar media. Then the cultures were incubated at 28°C for 24 to 72 hours. If there were no growth on the 14th day the cultures were discarded.

The ulcer was categorised as follows:

1. Non-severe corneal ulcer - when the ulcer had a diameter of less than 6 mm with ulceration of the superficial one-third and suppuration of the superficial two-thirds of the cornea, without either chance of perforation or scleral suppuration.

2. Severe corneal ulcer - when the ulcer had a diameter of 6 mm or more with ulceration and suppuration involving the deep one-third of cornea with the possibility of perforation and scleral suppuration [37,38]. If posterior corneal

abscess or endothelial plaque were present the ulcer was also categorised as severe.

Signs of improvement were as follows:

1. Blunting of the margins of ulcers.
2. Improvement of signs of inflammations.
3. Reduction in cellular infiltrate and oedema.
4. Reduction in corneal epithelial defect.
5. Signs of re-epithelialisation.
6. Reduction in anterior chamber hypopyon if present.
7. Decreased complaint of pain by the patient.

Signs of toxicity were as follows:

1. Patient's intolerance such as pain, burning sensation etc.
2. Swelling of the eye lids.
3. Increased conjunctival congestion and chemosis.
4. Conjunctival staining with flurescein.
5. Punctate corneal epithelial erosion.

The effect of the drug was expressed as follows:

1. No response, means all symptoms and signs remain the same as when recorded at presentation or getting worse such as increasing ulcer size, infiltration or hypopyon.
2. Healed, means there is scar formation with no epithelial defect stained with fluorescein, no infiltrate, no hypopyon and improvement in vision or vision no worse than baseline level.
3. Perforated.

Consecutive patients with suppurative corneal ulcers attending the National Institute of Ophthalmology & Hospital were examined clinically and corneal scrapings were taken by Kimura spatula for Gram staining, 10% Potassium Hydroxide test and culture. The patients was selected for study if there was microscopic observation of hyphal fragments either in the 10% KOH or in Gram stained smear.

After taking informed consent from the patient or from his or her guardian the eligible patients were treated in the first day 1 drop half hourly for three hours, then 1 hourly for 2 days, 2 hourly for 5 days and then 3 hourly for 2 weeks a total of 3 weeks treatment. The drops were given only in the waking hours. A log book was maintained for dispensing medicines to the patients. If there was no response by 5 days, clear cut signs of deterioration of ulcers or development of any untoward reactions the drug was withdrawn and the patient was managed as per standard hospital procedure. The eyes were examined daily and findings were recorded on the 2^{nd}, 5^{th}, 7^{th} day and anytime during the next 2 weeks. Each patient was given a discharge summary with the date on which he or she should come back to the hospital for follow-up. A post card was mailed 5 to 7 days prior to the due date as reminder to the patients. Even after this a few patients were failed to be seen and were considered as a lost to follow-up case.

CHAPTER: VI

RESULT

We studied 96 patients, of which 48 were allocated to the chlorhexidine gluconate 0.2% group and 48 to the natamycin 5% group (Table-2). 27 patients were classified as severe, with little prospect of recovery (although 3 severe ulcers were in fact healed beyond 21 days by chlorhexidine gluconate 0.2%). In chlorhexidine gluconate 0.2% group 1 non-severe ulcer (study no-59) was dropped out for follow-up leaving 47 patients in the study. In natamycin 5% group 1 non-severe ulcer (study no-40) was also dropped out for follow-up leaving 47 patients in the study. Other characteristics of the ulcers of the two treatment groups are given in Tables 5 to 12. Results of 10% KOH and Gram stain are shown in table-17.

None of the severe ulcers were healed either by chlorhexidine gluconate 0.2% or natamycin 5% by 21 days. Three severe ulcers were healed beyond 21 days by chlorhexidine gluconate 0.2%, 1 after 26 days, 1 after 37 days, and 1 after 60 days, but none by natamycin 5%. The overall healing rates at 21 days were 43.75% and 31.25% by chlorhexidine gluconate 0.2% and natamycin 5% respectively shown in table-3 and graph-2. In the case of only non-severe ulcers the healing rates were 67.74% and 41.66% by chlorhexidine gluconate 0.2% and natamycin 5% respectively shown in table-4 and graph-3.

Out of 96 patients we could follow-up 94 patients to see the ultimate fate of the ulcers. 36 healed by 21 days and 55 had no response, 3 severe ulcers were improving but healed beyond 21 days by 0.2% chlorhexidine gluconate and 2 improving non-severe ulcers were dropped out for follow-up, 1 from each group (Table-3).

The right eye was more affected than the left eye (Table-5). Both male and female were affected in the percentage of 75% and 25% respectively (Table-6). Corneal ulcer mostly occurred in the 21 to 50 age group, which accounts about 74.98% of total patients (Table-7). About 36.45% affected people were farmers, others were outdoor working groups i.e. those who are very much prone to trauma (Table-8). Trauma was the main predisposing factor for fungal keratitis, accounting for about 57.29% (Table-9). Some patients had used steroids, antibiotics etc. in the same eye. Some of them had used native medicines, even snail juice and are shown in Table-10. About 20.83% patients attended the cornea clinic within the 1st week of illness and 35.41% patients by the 2nd week of illness, as shown in Table-11. Visual

acuities after healing are shown in Table-12. Visual acuity prior to corneal scraping are shown in table-13. Visual Acuity after healing of corneal ulcers with 0.2% chlorhexidine is shown table-14 and with natamycin 5% is shown in table-15. Ultimate fate of the corneal ulcers which were not heal by 21 days are shown in table-16.

Table-2

Patients randomised to 2 treatment groups in the randomised trial of chlorhexidine gluconate 0.2% and natamycin 5% done in the National Institute of Ophthalmology, Dhaka, Bangladesh.

Treatment groups	Non-severe	Severe	Total
Chlorhexidine 0.2%	32	16	48
Natamycin 5%	37	11	48
Total	**69**	**27**	**96**

Table-3

Results at 21 days of antifungal drugs in the randomised trial of chlorhexidine gluconate 0.2% and natamycin 5% done in the National Institute of Ophthalmology, Dhaka,, Bangladesh.

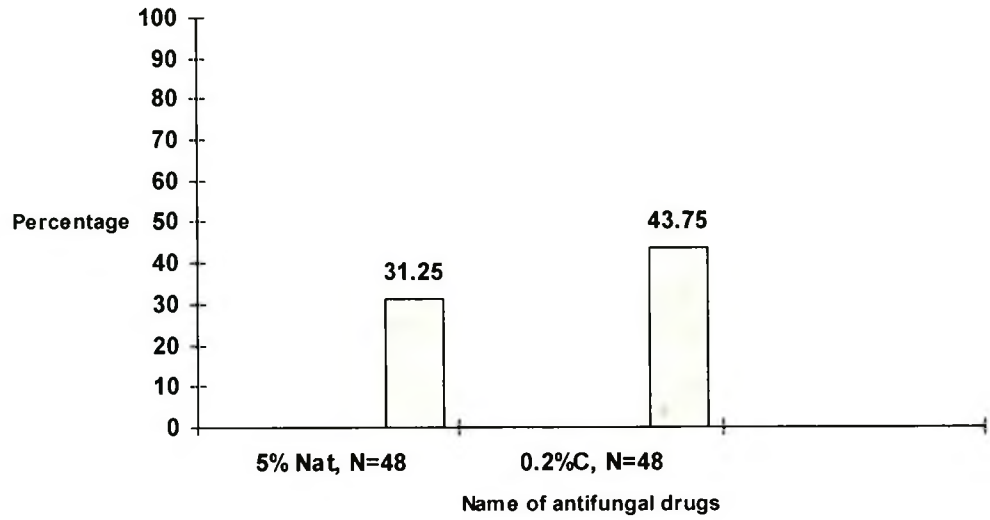
Name of drugs	No. of patients	Ulcers healed by 21 days	Drop out	No response	Improving but not healed by 3 week
0.2% chlorhexidine	48	21 (43.75%)	01**	23 (47.97%)	03*
5% natamycin	48	15 (31.25%)	01**	32 (66.66%)	00

*Eventually healed, they were all severe ulcers.

**Two improving non-severe ulcers were dropped out from each group.

Graph-2

Percentage of overall healed fungal keratitis at 21 days in the randomised trial of chlorhexidine gluconate 0.2% and natamycin 5% done in the National Institute of Ophthalmology, Dhaka, Bangladesh.



C = chlorhexidine

Nat = natamycin

N = total number of patients

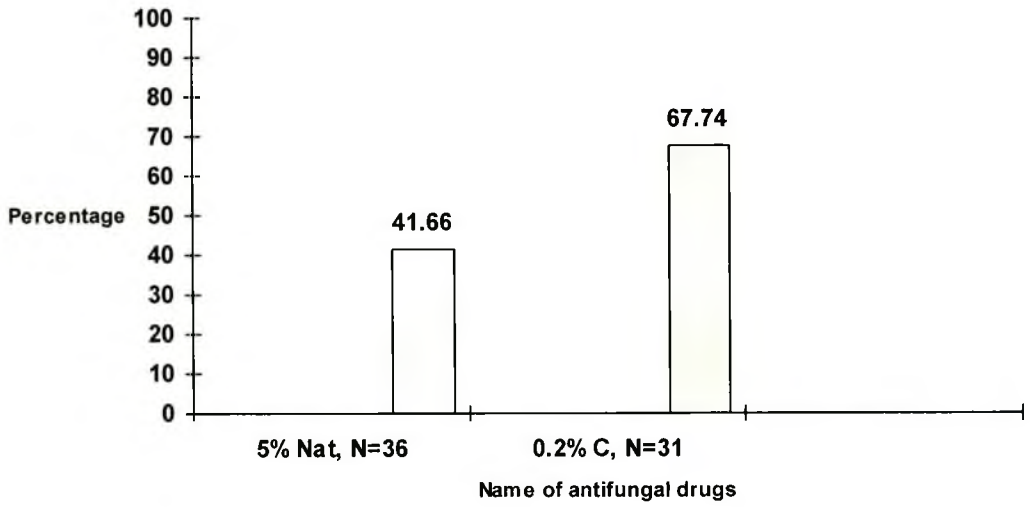
Table-4

Percentage of healed non-severe fungal keratitis at 21 days in the randomised trial of chlorhexidine gluconate 0.2% and natamycin 5% done in the National Institute of Ophthalmology, Dhaka, Bangladesh.

Name of drugs	No. of Non-severe ulcers	Ulcers healed by 21 days	Drop out	No response
0.2% chlorhexidine	32	21 (67.74%)	01	10 (32.25%)
5% natamycin	37	15 (41.66%)	01	21 (58.33%)

Graph-3

Percentage of healed non-severe fungal keratitis at 21 days in the randomised trial of chlorhexidine gluconate 0.2% and natamycin 5% done in the National Institute of Ophthalmology, Dhaka, Bangladesh.



C = chlorhexidine

Nat = natamycin

N = total number of patients

Table-5

Eye affected in the randomised trial of chlorhexidine gluconate 0.2% and natamycin 5% done in the National Institute of Ophthalmology, Dhaka, Bangladesh.

Eye	**0.2% chlorhexidine**	**5% natamycin**	**Total number**	**Percentage**
Right eye	27	28	55	57.29%
Left eye	21	20	41	42.70%

Table-6

Sex distribution of patients in the randomised trial of chlorhexidine gluconate 0.2% and natamycin 5% done in the National Institute of Ophthalmology, Dhaka, Bangladesh.

Sex	0.2% chlorhexidine	5% natamycin	Total no. of patients	Percentage
Male	34	38	72	75.00%
Female	14	10	24	25.00%

Table-7

Age distribution of patients in the randomised trial of chlorhexidine gluconate 0.2% and natamycin 5% done in the National Institute of Ophthalmology, Dhaka, Bangladesh.

Age (Years)	0.2% chlorhexidine	5% natamycin	Total number	Percentage
0-10	00	01	01	1.04%
11-20	03	04	07	7.29%
21-30	16	18	34	35.41%
31-40	11	10	21	21.87%
41-50	10	07	17	17.70%
51-60	04	05	09	9.37%
61-70	03	03	06	6.25%
71-80	01	00	01	1.04%

Table-8

Occupation of patients in the randomised trial of chlorhexidine gluconate 0.2% and natamycin 5% done in the National Institute of Ophthalmology, Dhaka, Bangladesh.

Occupations	0.2% chlorhexidine	5% natamycin	No. of patients	Percentage
Farmer	18	17	35	36.45%
Labourer	05	07	12	12.50%
Businessman	03	02	05	5.20%
Student	02	02	04	4.16%
House wife	12	11	23	23.95%
Service	02	03	05	5.20%
Mechanic	02	04	06	6.25%
Driver	01	00	01	1.04%
Carpenter	01	01	02	2.08%
Rickshaw puller	02	01	03	3.12%

Table-9

Predisposing factors of fungal keratitis in the randomised trial of chlorhexidine gluconate 0.2% and natamycin 5% done in the National Institute of Ophthalmology, Dhaka, Bangladesh.

Predisposing factors		**0.2% chlorhexidine**	**5% natamycin**	**Total no. of patients**	**Percentage**
Trauma		30	25	55	57.29%
	Foreign body (Unknown)	04	03	07	7.29%
	Straw	01	01	02	2.08%
	Finger nail	01	01	02	2.08%
	Bamboo	01	01	02	2.08%
	Heat	00	01	01	1.04%
	Stone	01	01	02	2.04%
	Soil	01	01	02	2.04%
	Thorn	01	01	02	2.04%
	Wood	01	02	03	3.12%
	Paddy grain	10	08	18	18.75%
	Insect	04	04	08	8.33%
	Sand	05	01	06	6.25%
No history of trauma		17	24	41	42.70%

Table-10

Medications prior to corneal scraping in the randomised trial of chlorhexidine gluconate 0.2% and natamycin 5% done in the National Institute of Ophthalmology, Dhaka, Bangladesh.

Name of drugs	0.2% chlorhexidine	5% natamycin	No. of patients	Percentage
Antibiotic	27	23	50	52.08%
Steroids	01	02	03	3.12%
Antifungal	00	01	01	1.04%
Snail juice	01	01	02	2.08%
Homeopathic eye drops	01	01	02	2.08%
Eye drops (Unknown)	13	13	26	27.08%
Antibiotic & antifungal	02	03	05	5.20%
Steroid & antiviral	00	01	01	1.04%
Antibiotic & steroid	01	01	02	2.08%
Antibiotic & antiviral	01	01	02	2.08%
Antibiotic, antifungal & steroid	00	01	01	1.04%
None	01	00	01	1.04%

Table-11

Duration of illness prior to corneal scraping in the randomised trial of chlorhexidine gluconate 0.2% and natamycin 5% done in the National Institute of Ophthalmology, Dhaka, Bangladesh.

Duration (Days)	0.2% chlorhexidine	5% natamycin	No. of patients	Percentage
1 to 7	12	08	20	20.83%
8 to 14	16	18	34	35.41%
15 to 21	14	11	25	26.04%
22 to 30	05	11	16	16.66%
31 to 60	01	00	01	1.04%

Table-12

Visual acuity of patients after healing of corneal ulcers treated with chlorhexidine gluconate 0.2% and natamycin 5% in the randomised trial of chlorhexidine gluconate 0.2% and natamycin 5% done in the National Institute of Ophthalmology, Dhaka, Bangladesh.

Visual acuity	0.2% chlorhexidine	5% natamycin
***Non-severe ulcers**		
6/6	01	01
6/9	02	02
6/18	08	02
6/24	03	01
6/36	04	02
6/60	01	02
C.F.	01	02
H.M.	01	02
P.L.	00	01
Grand total	21	15
****Severe ulcers**		
6/60	01	00
3/60	01	00
3/60	01	00
Total	03	00

*Non-severe ulcers healed by 21 days.
**Severe ulcers healed beyond 21 days.

Table-13

Visual acuity of patients Prior to corneal scraping of corneal ulcers treated with chlorhexidine gluconate 0.2% and natamycin 5% in the randomised trial of chlorhexidine gluconate 0.2% and natamycin 5% done in the National Institute of Ophthalmology, Dhaka, Bangladesh.

Visual acuity	0.2% chlorhexidine	5% natamycin
Non-severe ulcers		
6/6	01	01
6/9	02	03
6/18	11	08
6/24	06	03
6/36	06	08
6/60	02	05
C.F.	02	04
H.M.	02	04
P.L.	00	01
Sub-total	32	37
Severe ulcers		
6/60	02	02
3/60	02	01
3/60	02	02
H.M	03	02
C.F	04	02
P.L	03	02
Sub-total	16	11
Grand Total	**48**	**48**

Table-14

Visual acuity of patients after healing of corneal ulcers treated with chlorhexidine gluconate 0.2% in the randomised trial of chlorhexidine gluconate 0.2% and natamycin 5% done in the National Institute of Ophthalmology, Dhaka, Bangladesh.

Study No.	Initial Vision	Final Vision
***Non-severe ulcers**		
02	6/60	6/24
04	6/24	6/18
10	6/60	6/36
13	6/9	6/6
33	6/60	6/18
36	6/36	6/18
38	H.M	H.M
46	6/24	6/18
49	6/60	6/18
50	6/12	6/9
55	6/60	6/24
60	6/36	6/18
61	6/60	6/36
66	C.F-3 feet	6/18
72	6/18	6/9
75	6/36	6/18
76	6/60	6/24
80	6/60	6/36
86	H.M	6/60
92	H.M	6/36
94	H.M	C.F-3 feet
****Severe ulcers**		
08	6/60	6/60
25	C.F-3 feet	3/60
44	H.M	3/60

*Non-severe ulcers healed by 21 days.

**Severe ulcers healed beyond 21 days.

Table-15

Visual acuity of patients after healing of corneal ulcers treated with natamycin 5% in the randomised trial of chlorhexidine gluconate 0.2% and natamycin 5% done in the National Institute of Ophthalmology, Dhaka, Bangladesh.

Study No.	Initial Vision	Final Vision
***Non-severe ulcers**		
03	6/24	6/9
18	6/24	6/6
22	6/60	6/9
31	C.F -1 foot	6/60
39	6/36	6/24
41	H.M	C.F-3 feet
42	C.F-1 foot	6/60
51	6/60	6/18
56	H.M	C.F-1 foot
64	6/36	6/18
79	6/60	6/36
85	H.M	H.M
87	6/60	6/36
91	PL	PL
95	PL	H.M

Table-16

Ultimate fate of Ulcers except those healed by 21 days, in the randomised trial of chlorhexidine gluconate 0.2% and natamycin 5% done in the National Institute of Ophthalmology, Dhaka, Bangladesh.

Treatment Groups	**No. of Patients**	**Healed beyond 21 days**	**Healed by alternate treatment**	**Anterior staphyloma**	**Adherent leucoma**	**Penetratin kerato plasty**	**Enucleation**
Ulcers not Severe							
0.2% chlorhexidine	10	00	03	02	04	01	00
5% natamycin	21	00	07	05	06	03	00
Severe ulcers							
0.2% chlorhexidine	16	03	03	05	02	02	01
2.5% natamycin	11	00	02	06	01	01	01
Total	**58**	**03**	**15**	**18**	**13**	**07**	**02**

* 2 patients Sl. No. 40 & 59 lost for follow up.
* 36 patients were healed by 21 days

Table-17

Results of Gram stain and potassium hydroxide (KOH) tests in the randomised trial of chlorhexidine gluconate 0.2% and natamycin 5% done in the National Institute of Ophthalmology, Dhaka, Bangladesh.

Name of tests	0.2% chlorhexidine	5% natamycin	Total no. of patients
Gram Positive	37	39	76
Gram Negative	11	09	20
KOH Positive	46	44	90
KOH Nagative	02	04	06

CHAPTER: VII

DISCUSSION

7.1. Characteristics of patients

As mycotic keratitis is very common in tropical poor developing countries and fungal keratitis affects poor agriculture workers, so the use of a drug such as econazole is not feasible.

Males from 30 to 50 years of age are more affected than any other age groups. Outdoor workers are more affected than extreme age groups who are not working outside and are not prone to develop trauma. About 57.29% of patients give a history of trauma, although some patients some times forget about trivial traumas. As people were more aware of eye care, there were fewer patients who used steroids and native medicines prior to corneal scrapings.

7.2. The clinical results

In a study it was shown that chlorhexidine gluconate 0.2% may be superior to povidone iodine 5% and equal to econazole 1% in producing cure of fungal corneal ulcers. The cured ulcer were 91%, 20% and 87.5% in chlorhexidine gluconate 0.2%, povidone iodine 5% and econazole 1% respectively[80].

In a randomised trial of different concentrations chlorhexidine gluconate and natamycin 5%, it was shown that chlorhexidine may be superior to natamycin 5% and the effect appears to improve with increasing concentration up to 0.2%[81]. It is possible that 0.3% or even higher concentrations would give superior results without toxicity to the cornea, but this has not been tested clinically. The proportions cured at 21 days with chlorhexidine gluconate 0.2% and natamycin 5% were 83.3% and 50% respectively[81].

In this randomised trial of chlorhexidine gluconate 0.2% and natamycin 5%, it is shown that chlorhexidine gluconate 0.2% may be superior to natamycin 5% and the proportions cured in non-severe ulcers at 21 days were 67.74% and 41.66% respectively. The severe ulcers were not healed at 21 days by chlorhexidine

gluconate 0.2% and natamycin 5%. Although 3 were cured by chlorhexidine gluconate 0.2% beyond 21 days and none by natamycin 5%.

The patients' tolerance was good. The drugs were not discontinued in any patient due to allergy or any other problems. No patient complained about burning, itching or tearing due to any one of these medications. In one patient in punctate epitheliopathy of the cornea was seen with chlorhexidine 0.2%. It appeared that the medication had been given more frequently than advised and the staining punctate lesions disappeared when the frequency of administration was reduced to the correct 3 hourly regime. There were no signs of drug intolerance like excoriation and discoloration of skin or contact dermatitis etc. after 3 weeks of application. No symblepharon was noticed, no evidence of follicles or papillary hypertrophy, no other untoward systemic complications were found.

7.3. Properties of chlorhexidine

Chlorhexidine gluconate is effective against fungal keratitis, it has already been recommended for the treatment of acanthamoeba keratitis and in vitro it shows good response against bacteria. It is well known to be effective against a range of Gram positive and Gram negative organisms. It is being used for the treatment of acanthamoeba keratitis[39]. It is effective against Chlamydia trachomatis[23]. Chlorhexidine gluconate is already used as a preservative in many eye preparations and is therefore approved for use in the human eye. It has been used in several clinical situations for about 40 years. These include sterilisation of the skin, prevention of sepsis in wounds and burns, prevention of urinary tract infections, especially in catheterised patients, and antisepsis in practical obstetrics, including vaginal washing with 0.2% chlorhexidine solution. Chlorhexidine gluconate is regarded as the most effective antimicrobial for oral use. The standard preparation is a 0.2% mouth wash, and it has been available for more than 20 years in some countries. The extensive literature on its antimicrobial properties, applications and safety has been reviewed by Denton[16,79].

When chlorhexidine was first applied to the sterilisation of soft contact lenses, a number of studies of possible toxicity to animal eyes were carried out. For example, Gasset and Ishii found no detectable changes from applications of solutions up to 2% to rabbit eyes twice daily for 7 days[35]. Aqueous chlorhexidine solutions were evaluated for retardation of epithelial regeneration after experimental corneal abrasions[59]. While irrigation with concentrations of 2% or 4% significantly slowed

the healing rate, concentrations of 1% or less did not statistically slow healing. It is important to stress that some preparations of chlorhexidine may contain detergents. These should be avoided as they are irritant to the eye.

It is suggested that if fungi are seen on Gram staining or potassium hydroxide mount, chlorhexidine gluconate might be a useful first line agent.

In considering chlorhexidine digluconate as a treatment for fungal keratitis in developing countries, where mixed infections may be common and laboratory facilities are not always available, an advantage will be its wide antimicrobial activity. Chlorhexidine may be recommended as a single antimicrobial agent against bacteria, fungus and acanthamoeba in situations where specific antibiotics or antifungal agents cannot be obtained.

7.4. Future work

1. A clinical trial of chlorhexidine as a primary treatment against any suppurative keratitis, including bacteria, would be valuable for the situation in developing countries. The second arm of the trial could use a combination of an antibiotic and specific antifungal agent.

2. It would be valuable to assess the use of chlorhexidine by paramedical workers as a prophylaxis in minor injuries or infections, to prevent severe corneal ulcers.

CHAPTER: VIII

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CHAPTER: IX

APPENDIX

Appendix no. 1.

Literature review of micro-organisms.

Sl. no..	Reference	Fungus	Bacteria
1.	Rahman MM., Management of fungal corneal ulcer. Trans. Ophthalmic. Soc. Bangladesh. 1981; 9: 12-19. [58] Islam Eye Hospital, Dhaka, Bangladesh. Period 1979 to 1980. Total 508 cases.	Fungus-22.64%. (115 cases) Aspergillus (all)	
2.	Williams G., Billson F., Hussain R., Howlader SA., Islam N., McClellan K. Microbiological diagnosis of suppurative keratitis in Bangladesh. Br. J. Ophthalmol. 1987;71:315-321[44] Chittagong Eye Infirmary and Training Complex, Bangladesh. August 1983 &84. Total 33 cases, Culture positive 21 cases. Total identified cases-21. .	Fungus-one-third (7 cases.) A. fumigatus-1. A. ochraceus-1. Fusarium solani-1. Unidentified filamentous fungi-4 33.33%.	Bacteria-two-third. (14 cases.) Strept. pneumoniae Ps. aeruginosa 66.67%.

3.	Dunlop AAS; Wright ED; Howlader SA; Islam N.; Hussain R., Suppurative corneal infection in Bangladesh[45] Chittagong Eye Infirmary & Training Complex, Bangladesh Total 142 cases. Culture positive 116 cases. No organisms seen on gram stain & culture was negative in 15 cases (10.7%). 89 cases showed consistent microscopy & culture. 27 cases showed growth on culture but not seen on gram stain. 11 cases organisms seen on gram stain but culture was negative. Total identified cases-127.	Fungus-35.9%, (51 cases) Aspergillus-19,(13%,). A. fumigatus. A. flavus. Fusarium -10,(7%.). F. solani. F. dimerum. Curvularia- 9,(6%). C. fallax. Lasiodiplodia . theobromae-2. Scedosporium-1. Epicoccum-1. Cylindrocarpon vaginae-1. Candida albicans-1. Dichotomophthropsis nymphaerum-1. Fungal hyphae seen-3. Lost-3. 40.16%	Bacteria-53.5%, (76 cases.) Pseudomonas-24% Strept. pneumoniae-17% 59.84%.
4.	Williams G., McClellan K., Billson F. Suppurative keratitis in rural Bangladesh: the value of Gram stain in planning management. International Ophthalmol. 1991;15:131-135[46]. Chittagong Eye Infirmary & Training Complex Bangladesh. Total 127 cases. 107 cases was culture positive. 89 cases show both Gram stain & culture positive. In 20 cases (15%) gram stain & culture was negative. 18 cases were gram negative but culture positive. Total identified cases-107	Fungus 40.2%(Isolates) (43 cases.) Aspergillus-21. A. fumigatus. A. flavus. Fusarium-12. Lasidoplodia-2. Acremonium-1. Curvularia-1. Dichromophoropsis-1. Fungi (Unidentified)-5.	Bacteria 59.8%(Isolates) (64 cases.) Strept. pneumoniae Pseudomonas

5.	Liesegang TJ., Forster RK., Spectrum of microbial keratitis in South Florida. Am. J. Ophthalmol.1980; 90: 38-47[47] Between I January 1969 to 31 December 1977. .(9 Years) South Florida, USA. Total 663 cases, In 371 cases organisms were isolated, In 292 cases organisms were not identified. In 16 negative cultures definite organisms were seen with Gram stain & Giemsa stain.	Fungus- 35%(Isolates) (133 cases) Fusarium-82. (61%) F. solani-76 (57%). F. episphaeria-3. F. moniliforme-1. F. nivale-1. F. oxysporum-1. Aspergillus-6. A. fumigatus-3. A. flavus-3. Curvularia-8. C. senegalensis C. verruculosa C. pallescens Acremonium (Cephalosporium)-4 Melanconiales (Colletotrichum atramentum)-3. Alternaria-2. Lasiodiplodia theobromae-5. Penicillum-4. Paecilomyces-2. Petriellidum boydii-2 (Allescheriaboydii)- Geotrichum candidum.-1. Myrathecum-1 Volutella-1. Cylindrocarpon-1. Drechslera (Helminthosporium)-1. Cladosporium-1. Candida albicans-10..	Bacteria . 65%(Isolates) (238 cases). Ps. aeruginosa-31% Staph. aureus. Strept. pneumoniae Staph. epidermidis Proteus Corynebacterium.

6.	Robert H. Rosa,Jr., MD. Darlene Miller, MA., Eduardo C. Alfonso, MD. The Changing Spectrum of Fungal Keratitis in South Florida. Ophthalmology, Volume 101, November 6, June 1994[48] Bascom Palmer Eye Institute, Miami,. Florida. Between January 1982 & January 1992. (10 Years). (Four new Fungi not described previously in South Florida e.g. C.parapsilosis, A.terreus, C.tropicalis, and Trichosporon beigellii) One study from South Florida between 1959& 1977, total 19 years.	Total 125 Fungal keratitis. Fusarium-79.(62.2%) F. oxysporium-47. (37%). F. solani-30 (23.6%) F. moniliforme-1. (0.8%) Unspecified-1.(0.8%) Candida-16.(12.5%) C. parapsilosis-11. C. albicans-4. C. tropicalis-1. Curvularia-11(8.7%) C. senegalensis C. verruculosa. Aspergillus-5 (4%) A. terreus-2. A. fumigatus-1. A. flavus-1. A. glaucus-1. Paecilomyces-4 (3.2%) Acremonium-3 (2.5%) Cylindrocarpon-2 (1.6%) Lasiodiplodia theobromae-2(1.6%) Petriellidum boydii-1 Melanconiales -1. Drechslera-1 Rhizopus-1. Trichosporn beigellii-1 Fusarium sp. accounted for 76% of fungal keratitis. Fusarium solani-29%.	
7.	Thomas PA., Keratomycosis(mycotic keratitis) In: Hay RJ., ed. Bailliere's Clinical Tropical Medicine and Communicable Diseases International Practice and Research. Tropical Fungal Infections. Bailliere Tindall London 1989 Vol. 4. No. 1 pp 269-285.[54] Institute of Ophthalmology Jesoph Eye Hospital, Tiruchirapalli, India.	Fungus-30% Aspergillus Fusarium Curvularia Acremonium Penicillium	

8	McCurrach FE., MBBS, and Taylor HR, MD., FRCAO; Infectious keratitis; Current opinion in Ophthalmology, 1992, 3: 458-465.[14] India.	Fungus-50%	
9.	Rosa RH., Jr., MD. Miller D., MA., Alfonso EC., MD, The Changing Spectrum of Fungal Keratitis in South Florida, 0phthalmology Volume 101, November 6, June 1994.[48] India	Aspergillus sp.(27%-64%) Fusarium sp. (6%-32%). Penicillium sp. (2%-29%).	

10.	Upadhyay MP., Karmacharya PCD., Koirala S., Tuladhar NR., Bryan LE., Smolin G., Whitcher JP., Epidemiologic characteristics, predisposing factors and etiologic diagnosis of corneal ulceration in Nepal. Am. J. Ophthalmol. 1991; 111: 92-99[8] Tribhuban University Teaching Hospital, Kathmandu Nepal., Between September 1985 & August 1987. Total cases-405. Culture positive cases-324. Culture negative cases-81. Pure bacterial growth-256. Pure fungal growth-27. Mixed bacterial & fungal growth-41. Total identified cases-324.	Fungus-16.8% (68 cases.) Pure fungus-6.7% Aspergillus-31 (47%). A. fumigatus A. flavus A. restricuts A. ustus A. versicolor A. sydowii A. terreus A. oryzae Candida-9 (13.2%). C. krusei C. tropicalis C. pseudotropicalis C. parapsilosis C. guilliermondii Geotrichum candidum. Fusarium-8 (11.7%). F. oxysporum F. solani F. heterospermum F.nivale F.sporotrichoides F.tabacinum Cladosporium-4. C. chlorocephalum C. cladosporoides C. sphaerospermum Mucor sp. -3. M. globosus M. mucedo M. racemosus Lasiodiplodia-3. Curvularia-2, Collectotrichum-1. Nigrospora --1. Phialophora-1. Penicillium-1. Rhizopus-2. Sporotrichum-1. 21%	Bacteria-73.3% (297 cases) Pure bacteria-63.2% Strept. pneumoniae Staph. epidermidis Staph. aureus Corynebacterium xerosis Pseudomonas. 79%

11.	Ormerod LD. Causation and management of microbial keratitis in Subtropical Africa. Ophthalmology 1987; 94: 1662-1668[55] St. John's Eye Hospital, Soweto, South Africa. Between 1 March 1981 & 31 January 1982. (11 month). Total 120 patients with 131 episodes. Culture positive-85 cases. Culture negative-28 cases. Culture not available-12 cases. Culture not done-6 cases. Total identified cases-85.	Fungus-3.53% (3 cases) F. solani.-1 Aureobasidium pululans-1 Dichotomophthora portulacae-1.	Bacteria-96.47% (82cases). Staph. aureus Streptococcus Pseudomonas H. influenzae Proteus Klebsiella Enterobacter.
12.	Carmichael TR., Wolpert M., Koornof HJ. Corneal ulceration at an urban Africa hospital. Br. J. Ophthalmol. 1985; 69: 920-926[56] St. John's Eye Hospital, Baragwanath Hospital Soweto, South Africa. Between July 1982 & June 1983. Total 283 Corneal ulcer.(from 274 patients.) Micro-organisms isolated from 87 Eyes. Total identified cases-87	Fungus-7% (6 cases.) Curvularia-2 A. flavus-1 Fusarium-1 Candida-1 Phomaeupyrena-1	Bacteria-93% (81 cases.) Strept. pneumoniae Staph. aureus Ps. aeruginosa Moraxella
13.	Coster DJ., Wilhelmus K., Peacock J., Jones BR., Suppurative keratitis in London. VIth Congress of the European Soc. of Ophthalmol. 1981: pp 395-398.[57] Moorfields Eye Hospital, London. Between April 1978 & November 1979. Total 67 cases. In 41 cases organisms were found. In 26 cases no organisms were found. In 26 Gram negative 3 yield growth on culture. In 41 Gram positive 3 does not yield growth on culture. Total identified cases-41	Fungus-5%. (2 cases.) Candida-1 Cladosporium-1	Bacteria-95% (39 cases.) Staph. aureus. Strept. pneumoniae. Ps. aeruginosa Moraxella

14.	Asbell P., Stenson S. Ulcerative Keratitis; Survey of 30 years Laboratory Experience. Arch. Ophthalmol. 1982; 100: 77-80[12] New York University-Bellevue Medical Center.. Between 1950 to 1979 Total 30 Years. Total 677 cases. Organisms were identified from 547 cases. by Gram stain & culture. Positive cultures were obtained in 494 cases.	Fungus -1.7%. (only 9 cases) Candida-5 Fusarium-2 Nocardia-1 Cryptococcus-1 New York-1% Texas-17%. Florida-35%.	Bacteria -98.3% (538 cases.) Staphylococcus 48% Moraxella 16% Pseudomonas 8% Strept. pneumoniae 8% Proteus 2% and others.
15.	Gugnani HC., Talwar RS, Njoku-obi ANU, and Kodilinye HC; Mycotic Keratitis in Nigeria, A study of 21 cases; Brit. J. of Ophthal. (1976) 60, 607.[40] The study was limited to cases in which the corneal ulcers were strongly suggestive of a fungal infection. Total 26 patients. Fungus cultured in 21 patients.	Fungus (21 cases) Fusarium solani-12 A. fumigatus-4 A. flavus-1 Penicillium citrinum-2 P. expansum-1 Penicillium sp.-1	
16.	Cheung J., MD., Slomovic AR, MD, FRCSC; Microbial aetiology and predisposing factors among patients hospitalised for corneal ulceration, Can J. 0phthalmology-vol. 30, no. 5, 1995, 151-255[6]. The Toronto hospital. Total cases-95 Organisms grown-60 No growth-35	Fungus-0%	Bacteria-100% Coagulase-negative staphyloccus-30% Staph. aureus-23% Strep. pneumoniae-12% Strep. α hemolyticus-3% Gr. A Streptococcus-2% Enterococcus-2% Moraxella-7% Pseudo. aeruginosa-12% Proteus mirabilis-3% Klebsiella-3% Haemoph. influenzae-3% Serratia marcescens-2% Citrobacter diversus-2% E. coli-2% Mycobact. chelonae-2% Propionibacterium acnes-2%

17.	Tsiligianni AK, MD., Alfonso E., MD., and Forster RK, MD; Ulcerative Keratitis Associated With Contact Lens Wear, American Journal of Ophthalmology 108:64-67, July, 1989[51]. Bascom Palmer Institute, Miami, Florida. Total no. of cases-658 Contact lens wearers-196 Non-contact lens wearer-462 Culture positive-349 No cases of fungal keratitis were found in the contact lens wearer group.	Fungus-11.74% Fusarium sp-23 Curvularia sp-5 Others-12 Mixed-1	Bacteria-88.25% P. aeruginosa-132 Pseudomonas sp-5 Serratia marcescens-20 Proteus mirabilis-6 Morganella morganii-4 H. influenzae-5 N. gonorrhoea-3 Staph. aureus-42 Staph. epidermidis-18 Strep. pneumoniae-12 Streptococcus sp-10 Bacillus sp-3 Gram positive rods-12 Gram positive anaerobes-7 Other gram negative-18 Mixed-11
18	Ormerod LD, MD., Hertzmark E, MA., Gomez DS., BS., Stabiner RG., MS, Schanzlin DJ., MD., Smith RE., MD; Epidemiology of Microbial Keratitis in Southern California; Ophthalmology, October 1987, vol. 94 no. 10[52]. Southern California. Total cases-242 Culture-positive-186 Polymicrobial-62 Two organisms-52 Three organisms-10 Four organisms-1 Total no. of bacterial isolates-240 Total no. of fungal isolates-20	Fungus-10% C. albicans-9 Penicillium-3 Helminthosporium-3 Alternaria-2 Scopulariopsis-1 Cladosporium-1 A. flavus-1 Houston-17%. San Francisco-0% Boston-0%	Bacteria-90% Staph. aureus-41 Coagulase-negative staphyloccus-54 Strep. pneumoniae-27 Alpha-streptococci-17 Beta-streptococci-13 Gama-streptococci-4 Microaerophilic streptococci-1 Moraxella-14 Acenetobacter-2 Branhamella catarrhalis-1 B. subtilis Clostridium sp-1 P. aeruginosa-35 Pseudomonas sp-2 Proteus sp-8 E. coli-5 Klebsiella sp-3 Serratia sp-3 Citrobacter sp-2 Enterobacter sp-2 Alcaligenes faecalis-1 Aeromonas hydrophila-1 Eikenella corrodens-1 H. influenzae-1

19.	Hagan M., Wright E., Newman M., Dolin P., and Johnson G.; Causes of Suppurative Keratitis in Ghana; [41] Total no. of patients-199. Nothing cultured-85 cases. Organisms cultured-114 patients. Single organism cultured-103 cases. Gram positive & Gram negative-3 cases. Gram positive & fungus-4 cases. Gram negative & fungus-2 cases. Gram positive, Gram negative & 1 fungus-1 Gram positive, Gram negative & 2 fungus-1 Single bacteria-50 Mixed-8 Total-58 Single fungus-56 Mixed-8 Total-64.	Fungus alone-49.12% Mixed-56.14% F. solani-6 F. dimerum-1 Fusarium sp.-27 A. fumigatus-1 A. flavus-5 A. terreus-1 Aspergillus sp. -3 Pseudallescheria boydii-1 Cladosporium sp.-4 L. theobromiae-6 Trichosporon capitatum-1 Nigrospora sp-1 Candida parapsilosis-1 Curvularium fallax-2 Acremonium sp.-1 Phoma sp.-1 Dichotomophthoropsis sp.-1 Unidentified fungi-2	Bacteria alone-43.85% Mixed-50.87% S. pneumoniae-8 Streptococcus sp-3 Enterococcus faecalis-1 Corynebacterium sp-3 S. aureus-4 S. epidermidis-14 Propionibacterium acnes-1 Moraxella sp-4 H. influenzae-1 N. gonorrhoeae-2 Neisseria sp-1 P. aeruginosa-16 Pseudomonas sp-1 Enterobacter cloacae-2 Vibrio metschnikovii-1 Alcaligenes sp-1