Prevalence and genotyping of *Acanthamoeba* spp. in keratitis patients from a tertiary care centre of North India

Kirti Megha[1]

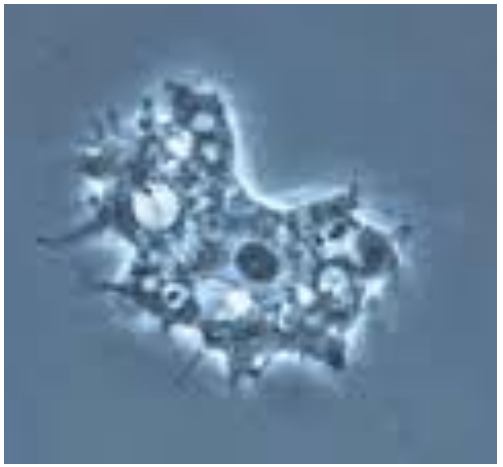
Sumeeta Khurana, [1] Rakesh Sehgal[1] and Amit Gupta[2]

Department of Medical Parasitology[1] and Ophthalmology[2]

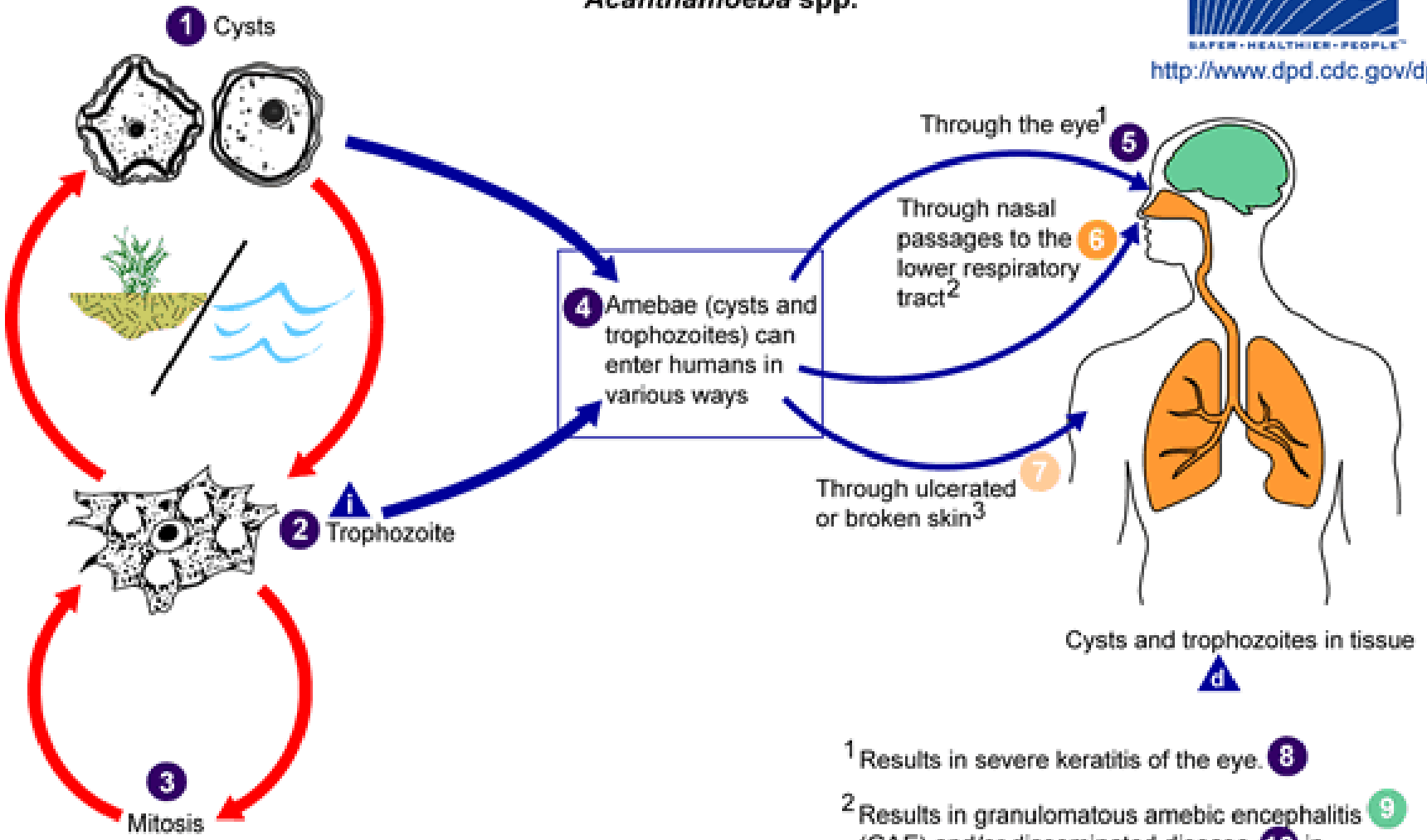
Postgraduate Institute of Medical Education and Research, Chandigarh, India

Introduction

- *Acanthamoeba* spp. are increasingly recognized as important microbes
- Ubiquitous free living protozoa, isolated from several habitats
- Clinical manifestations :
  - Granulomatus amoebic encephalitis
  - Cutaneous lesion and sinusitis
  - Acanthamoeba keratitis (AK)



Acanthamoeba spp.



i = Infective Stage

d = Diagnostic Stage

[1] Results in severe keratitis of the eye. 8

[2] Results in granulomatous amebic encephalitis 9 (GAE) and/or disseminated disease 10 in individuals with compromised immune systems.

[3] Results granulomatous amebic encephalitis 9 (GAE), disseminated disease 10, or skin lesions 11 in individuals with compromised immune systems.

CDC
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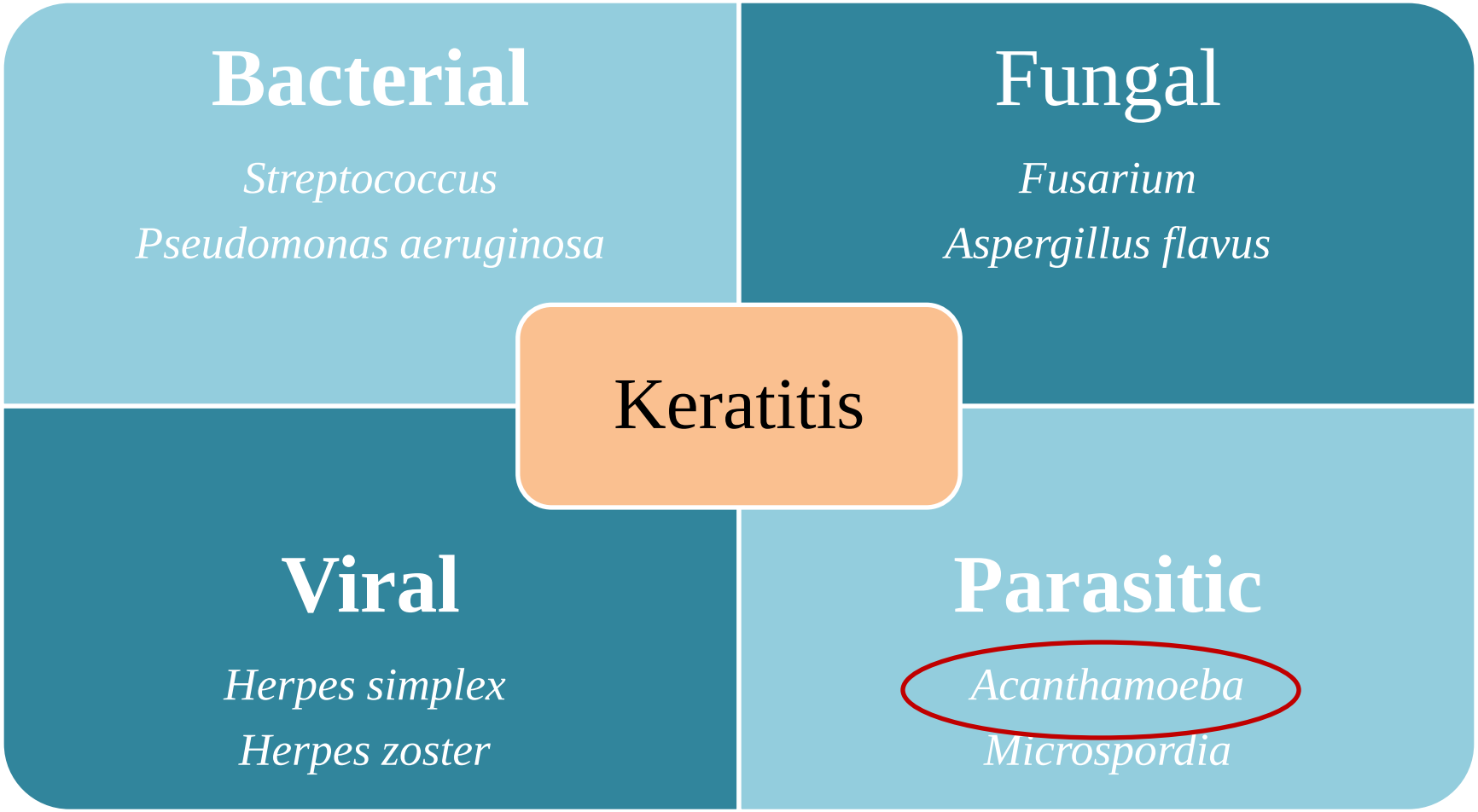
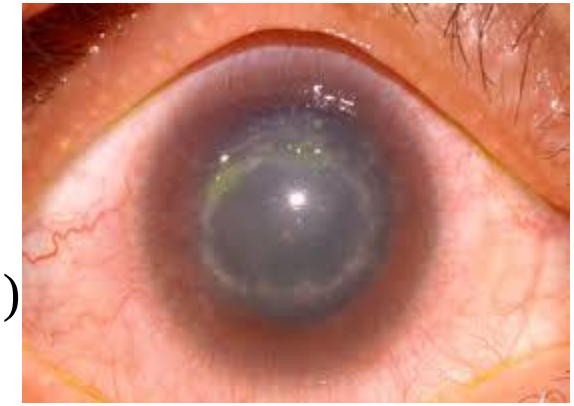
http://www.dpd.cdc.gov/dpdx


Bacterial
Streptococcus
Pseudomonas aeruginosa
Fungal
Fusarium
Aspergillus flavus
Keratitis
Viral
Herpes simplex
Herpes zoster
Parasitic
Acanthamoeba
Microspordia

Acanthamoeba keratitis



- *Acanthamoeba* can cause sight threatening - AK
- Developed countries - Contact lens wearer (< 80%)
- Developing countries - Corneal injuries
- India
  - Uncommonly related to contact lenses
  - Mostly occur after corneal trauma or exposure to contaminated water

Epidemiology

INCIDENCE - USA - 0.01
England - 0.19
Scotland - 1.49
Hong Kong - 0.33
Netherlands - 3.06
India - 0.9%

/10000contact lens

- At present -120 million people wearing contact lens in world
- USA-85% cases occurs in contact lens wearers

India scenario

L. V. Prasad Eye Institute, Hyderabad

Gunisha Pasricha *et al*., 2003

Microbiological diagnosis in 53 patients with microbial keratitis

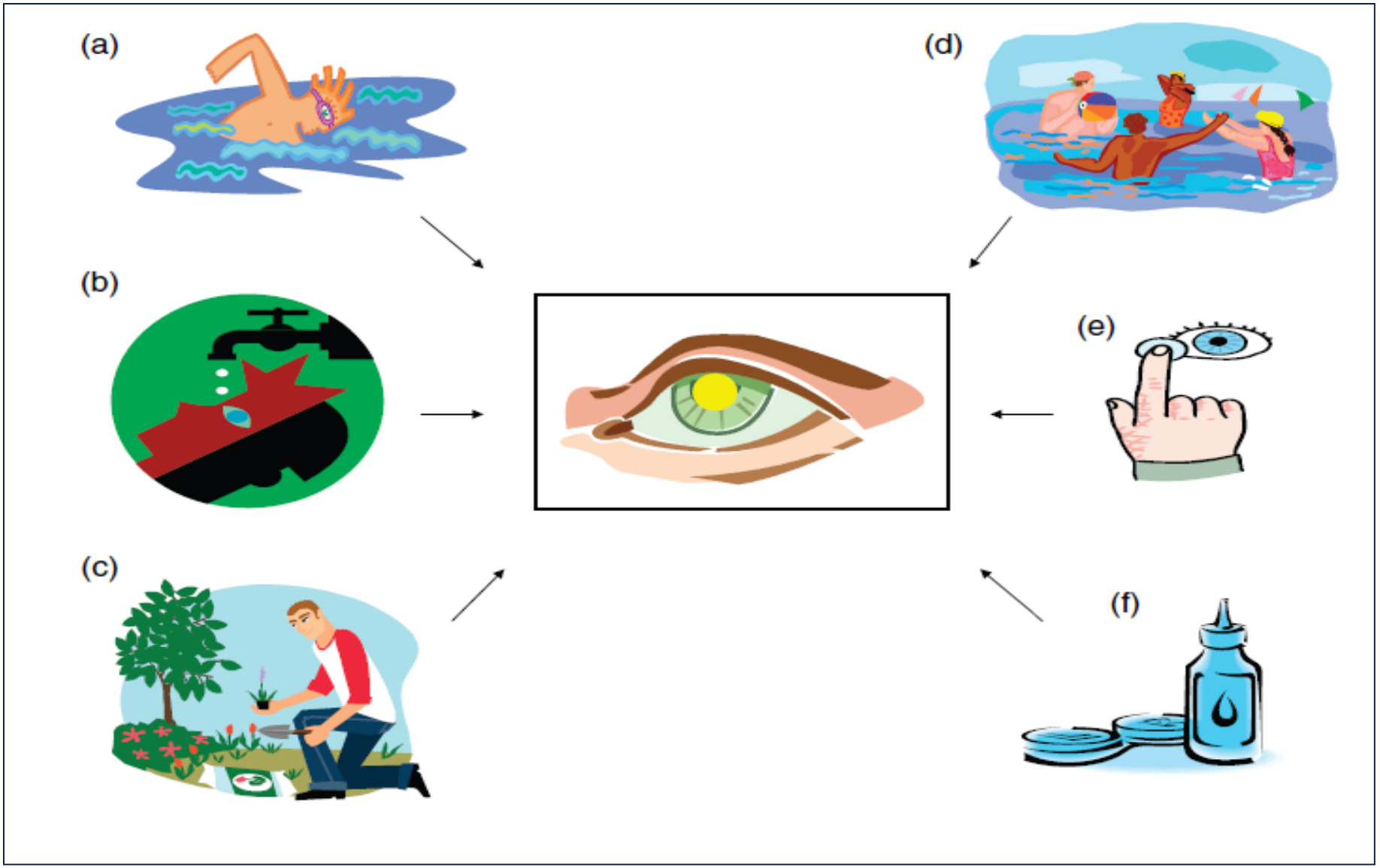
Microbiological diagnosis[a]	
Bacterial	17 (32.0)
Fungal	14 (26.4)
Acanthamoeba	7 (13.2)
Viral (HSV)	2 (03.8)
Bacterial + *Acanthamoeba*	1 (01.9)
Fungal + viral	1 (01.9)
Sterile (unknown)	11 (20.7)

[a] Based on culture of bacteria, fungi, or *Acanthamoeba* and antigen and/or DNA detection of herpes simplex virus (HSV) in corneal scrapings.

- Retrospective analysis of microbiology laboratory records at the Aravind Eye Hospital (Madurai) Lalitha *et al*., 2012

  - *Acanthamoeba* comprised 1% of all infectious keratitis cases and 2.8% of all culture-positive cases
  - 0.9% of the occurrence among contact lens wearers

Risk factors



Diagnosis

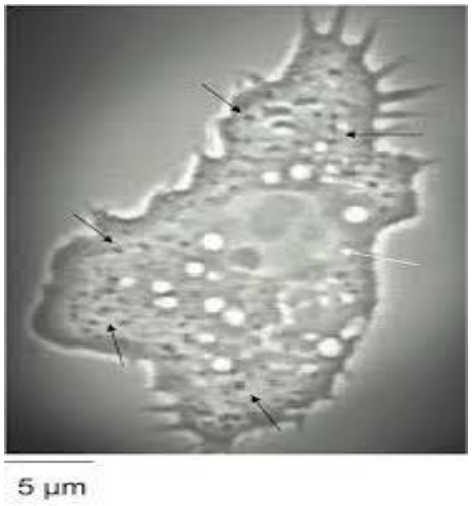
Technique	Sensitivity	Specificity	Reference
Microscopy	30-40%	100%	Yera *et al.*, 2006
NNA Culture	73.7%	100%	Boggild *et al.*, 2009
PCR	90%	100%	Boggild *et al.*, 2009
Real Time PCR	97%	100%	Thompson *et al.*, 2008

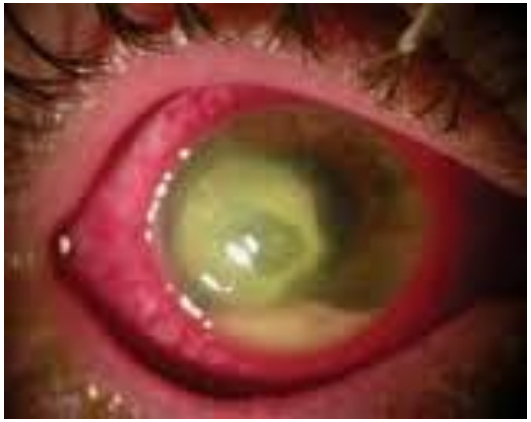
Genotyping

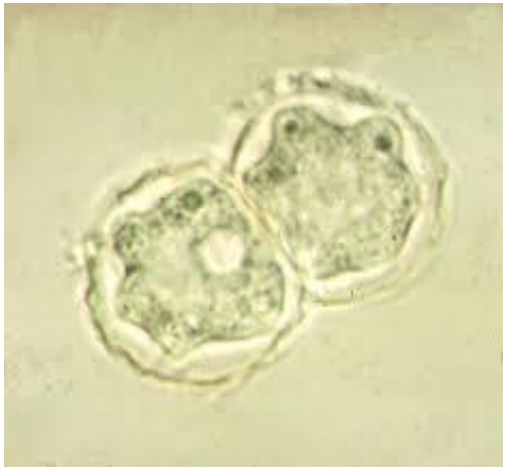
- Useful tool for studying taxonomic and epidemiological relationships
- Based on 18S rDNA gene sequences, the genus *Acanthamoeba* is divided into 19 genotypes (T1-T19)
- Subset of the ASA.S1 amplimer (463 bp) sequence has been accepted for genotyping by genus-specific primers JDP1-JDP2
- Targeting a highly variable region designated as diagnostic fragment 3 (DF3) within ASA.S1 by sequencing with primer 892C

Aim

Prevalence and genotyping of *Acanthamoeba* spp. in keratitis patients from a tertiary care centre of North India.

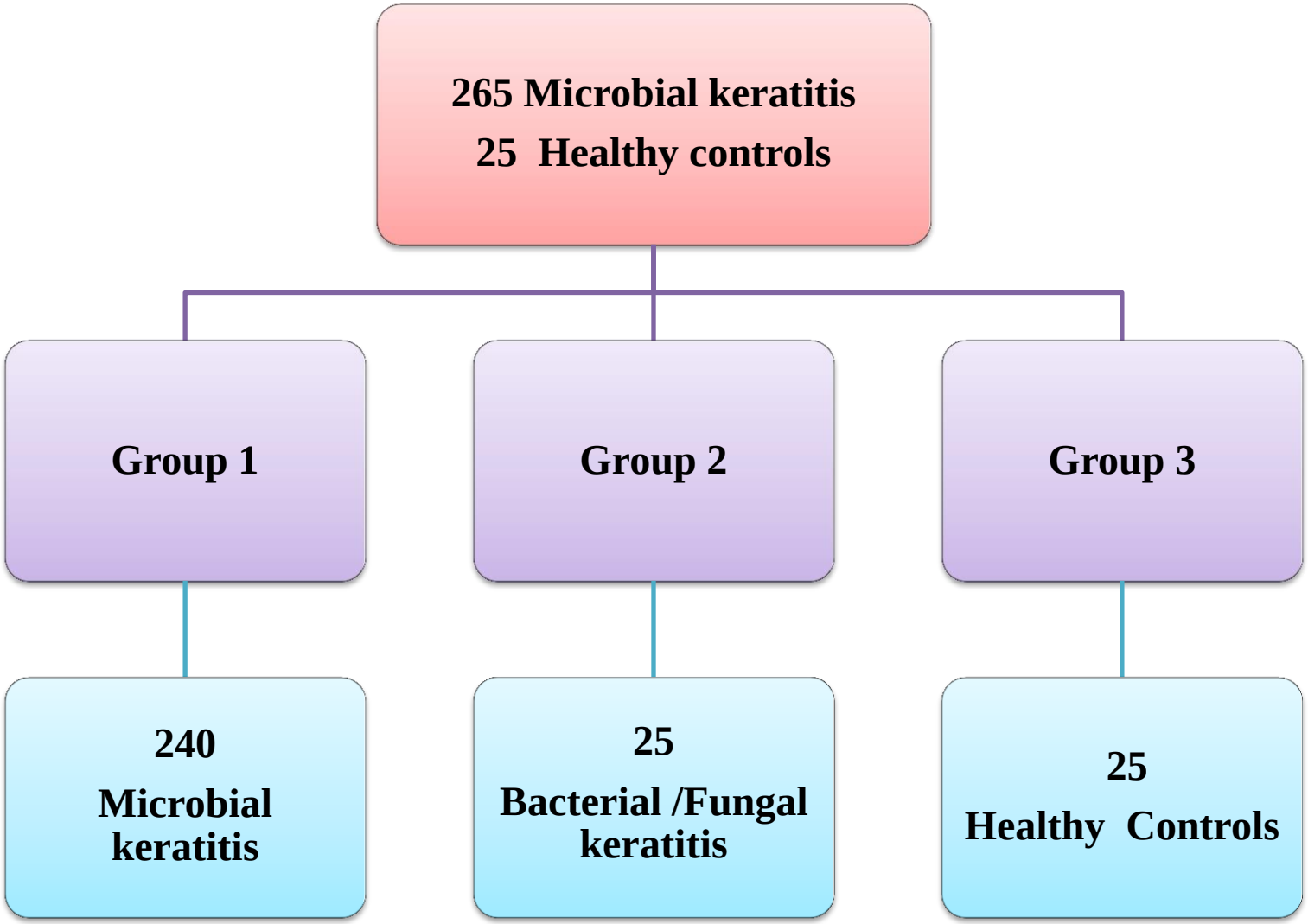






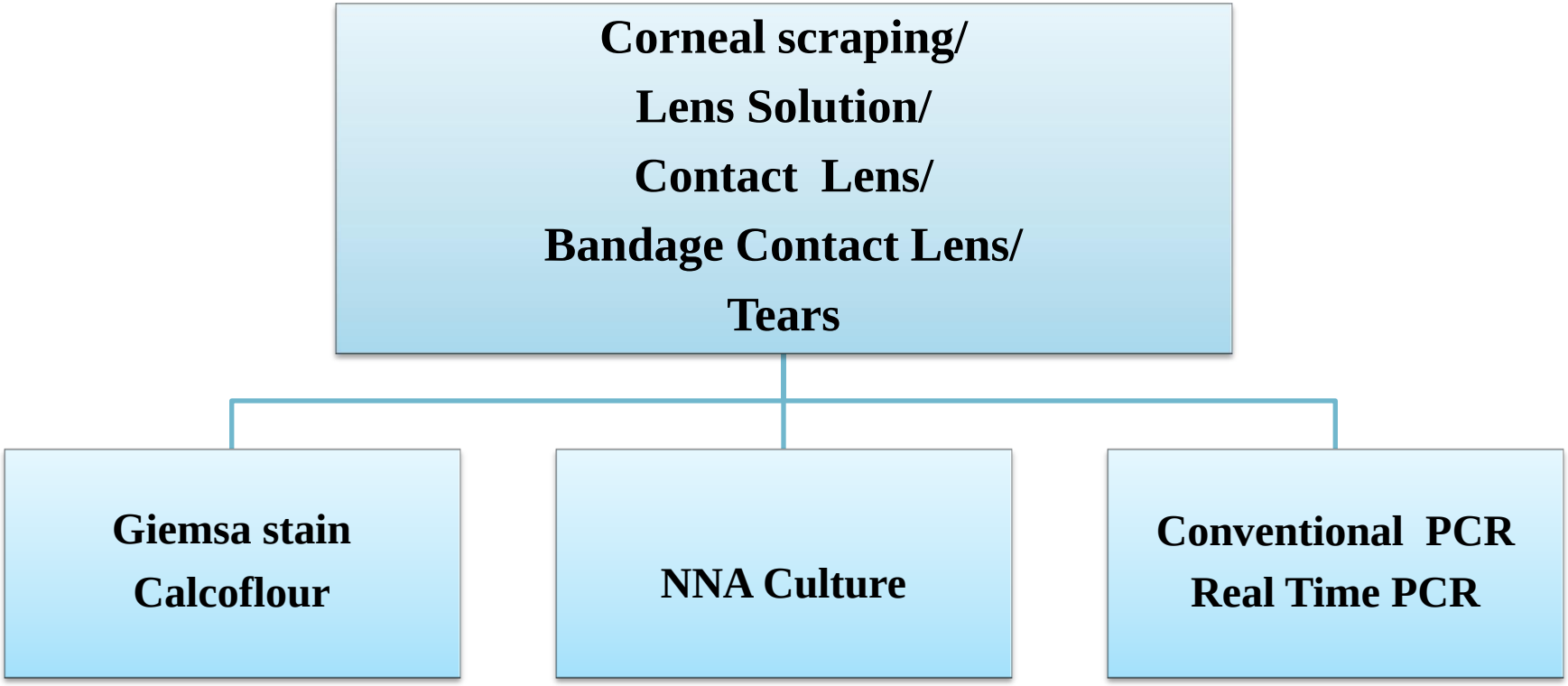
Material and methods

Patient Samples



Methods

Sample processing



Microscopy

- **Giemsa staining**

Methanol fixation

↓

Giemsa stain

↓

Observed under light microscope

- **Calcofluor Staining**

Methanol fixation

↓

Add drop of calcoflour solution

↓

Counterstained with 0.1% Evan's blue

↓

Fluorescent microscope (395 to 415 nm)

Culture Method

- **Non Nutrient Agar (NNA)**

Corneal scraping/Lens solution/Contact Lens/ BCL/washing from lens case/ Tears

↓

Directly inoculated onto culture plates overlaid with *E. coli*

↓

Incubated at 30^0C for 8 days

↓

Observed every 2 days using inverted microscope

Molecular diagnosis

- **DNA Extraction**
  - Phenol chloroform Isoamyl alcohol method

- **Polymerase Chain Reaction**
  - 18s rDNA of *Acanthamoeba*
  - JDP1- 5'-GGCCCAGATCGTTTACCGTGAA-3'
  - JDP2-5'-TCTCACAAGCTGCTAGGGAGTCA-3'
  - Amplicon - 500bp

Initial denaturation	**95 °C -7min**
Denaturation	95°C -1min
Annealing	60 °C – 1min
Extension	72 °C – 2 min
Final extension	72 °C - 10 min

Real Time-PCR

- 18s rDNA of *Acanthamoeba* was amplified with following primer pair-
- F 5’-CCCAGATCGTTTACCGTGAA-3’
- R 5’-TAAATA TTAATGCCCCCAACTATCC-3’
- Amplified product size of 180bp

Reaction mixture for Real time PCR	
Water	3µL
PCR primers, 10X conc.	2µL
SYBR green mix, 2X conc.	10µL
DNA	5µL

Real –Time PCR amplification conditions

Amplification (LightCycler 480)	
Preincubation	
95°C	10 min
Amplification	40 cycles
95°C	10s
62°C	15s
72s	10s
Melting curve	
95°C	10 s
67°C	15s
95°C	Continuous
Cooling	
40°C	30s

Determination of analytic sensitivity PCR and Real-time PCR

- Serial dilutions of gDNA
- Quantification of gDNA were determined by Nanodrop
- Initial conc was made to achieve 100 ng/µl
- 10 fold Serial dilutions : 100ng to 1fg

Analytical specificity
PCR and Real-time PCR

Fungal, bacterial

Tear samples of healthy control

P. vivax, *T. gondii*, *G. lambia*, *T. vaginalis*, *L. donovani* and *E. histolytica*

Assays only measure *Acanthamoeba*

Genetic characterization of *Acanthamoeba* isolates

- **Sequencing of the isolates**

  – Direct sequencing of PCR products containing DF3 were performed with sequencing primers

  892C (5’ GTCAGAGGTGAAATTCTTGG-3’)

  – Multiple Sequence aligned by Clustal W software

  – Phylogenetic tree was constructed using MEGA 6.06 software

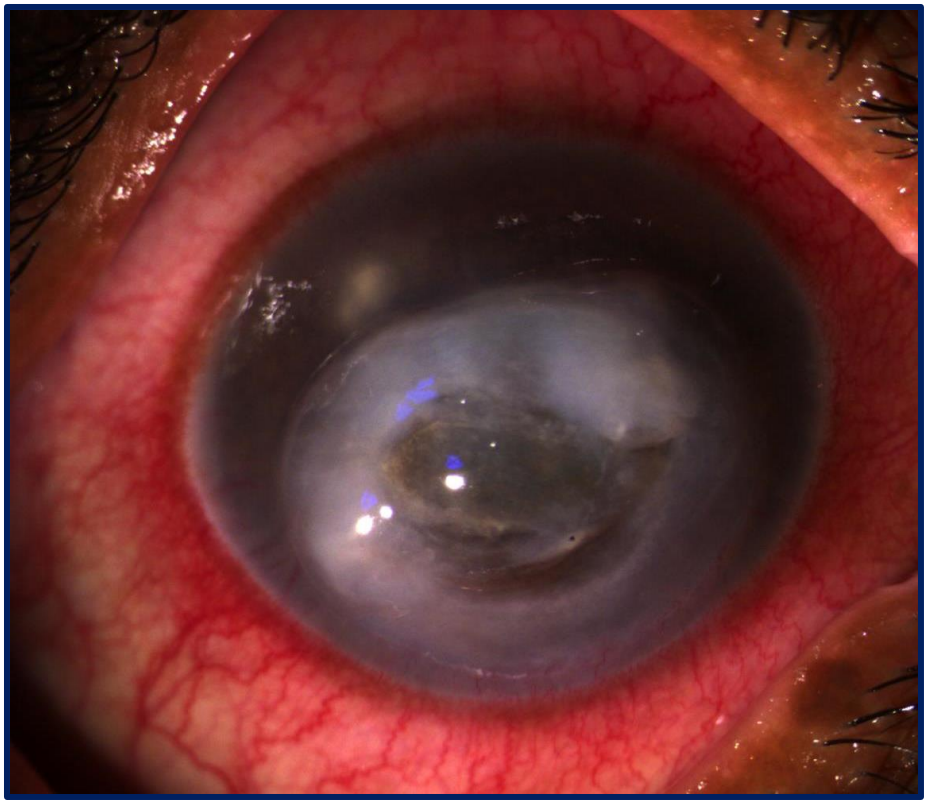
Results

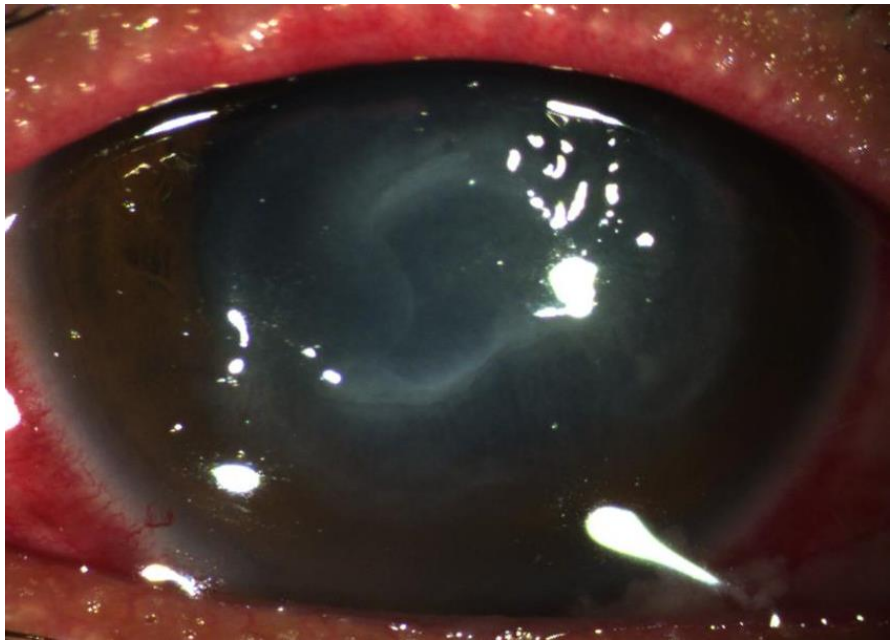
Samples	No of Patients	Microscopy	NNA Culture	PCR	Real –time PCR
Corneal scrapings	221 patients	1	3	3	3
Bandage contact lens	19 patients	0	1	1	1
Lens case	6 patients	0	1	1	1
Contact lens	9 patients	0	0	0	0
Lens solution	9patients	0	0	0	0
Tears (Healthy Control)	25 patients	0	0	0	0

Cases	Age/ Sex	Occupation	Signs & Symptoms	H/O	Contact Lens Wear	Opht. finding	Prediagnostic Treatment	Microbiological investigation	Parasitological Investigation	Postdiagnostic Treatment	Surgical intervention	Response to treatment
Case I	60/F	Agriculturist	DOV, Redness, watering ,photophobia Severe Pain in RE - 7 days	Trauma with wooden stick	No	perforated corneal ulcer4.5X6.5mm	Moxifloxacin 0.5% 6t/d Atropine 1% TDS	Gram Staining –Ve 10% KOH-ve	Microscopy NNA Culture Positive PCR Positive	**Polymyxin** (10,000 IU/ml) 4 hrly moxifloxacin 0.5% 6t/d	TPK	Yes
Case II	72/M	Agriculturist	Redness, Pain watering RE X 2 months	Trauma with vegetative matter	No	Central corneal ulcer 1.6 X5mm	Acyclovir cefazolin 5% 6t/d amikacin 1.4% 6t/d atropine 1% TDS	gram stain and 10 % KOH –ve	NNA Culture positive PCR positive	**Polymyxin** (10,000 IU/ml) 4hrly X 2 Months moxifloxacin 0.5% 6t/d atropine 1% TDS	No	Yes
Case III	27/M	Taxi Driver	c/o pain Redness DOV in RE x 1week	No	No	ring infiltrate with epithelial defect and hypopyon	cefazolin 5% 6t/d amikacin 1.4% 6t/d natamet 5% 1rly	gram stain and 10% KOH - ve	NNA Culture positive PCR positive	**Polymyxin** (10,000 IU/ml) 4hrly 8 weeks cefazolin 5% 1hrly	No	Yes

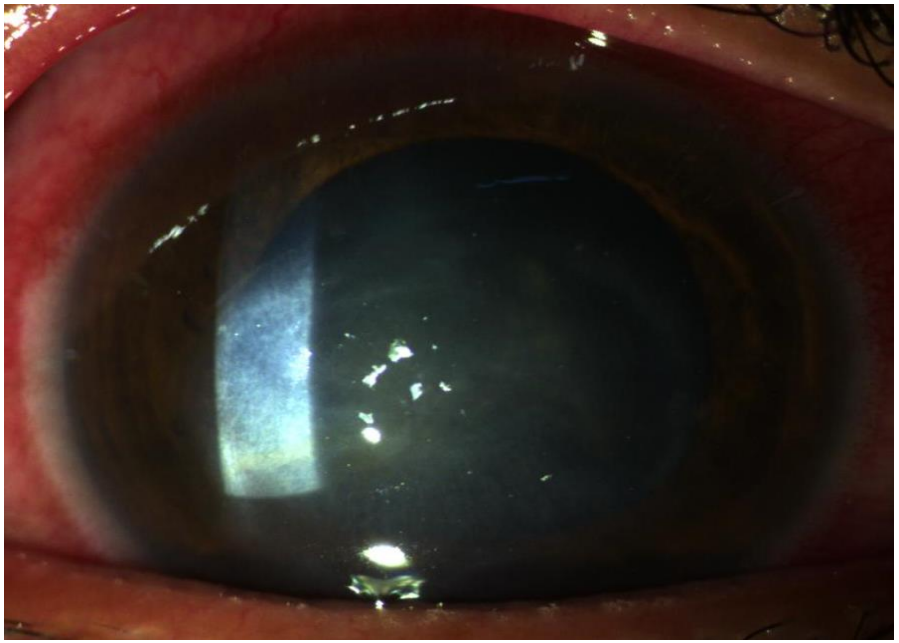
Cases	Age/ Sex	Occupation	Signs & Symptoms	H/O	Contact Lens Wear	Opht. finding	Prediagnostic Treatment	Microbiological investigation	Parasitological Investigation	Postdiagnostic Treatment	Surgical intervention	Response to treatment
Case IV	22/M	Pvt. Job	c/o Redness watering blurring of vision BE x 2 days	No	H/o using contact lens B/E while sleeping H/o keeping contact lens in tap water contact lenses non available	Corneal infiltrates and epithelial defect	I.V. vancomycin 1gm BD x 3 days cefazolin 5% 6t/d amikacin 1.4% 6t/d Natamet 5% hourly	gram stain and 10 % KOH - ve	NNA Culture positive PCR positive	**Polymyxin** (10,000 IU/ml) 4 hr;ly amikacin 1.4% 1hrly moxifloxacin 0.5% 6t/d	No	Yes
Case V	23/M	Pvt . Job	C/O DOV Redness Pain	H/O C3R surgery	No	Multiple epithelial pearls BCL were in placed	moxifloxacin 0.5% 6t/d cefazolin 5% 6t/d amikacin 1.4% 6t/d		NNA Culture positive PCR positive	Polymyxin (10,000 IU/ml) 4 hrly amikacin 1.4% 1hrly moxifloxacin 0.5% 6t/d	No	Yes

Clinical appearance of AK

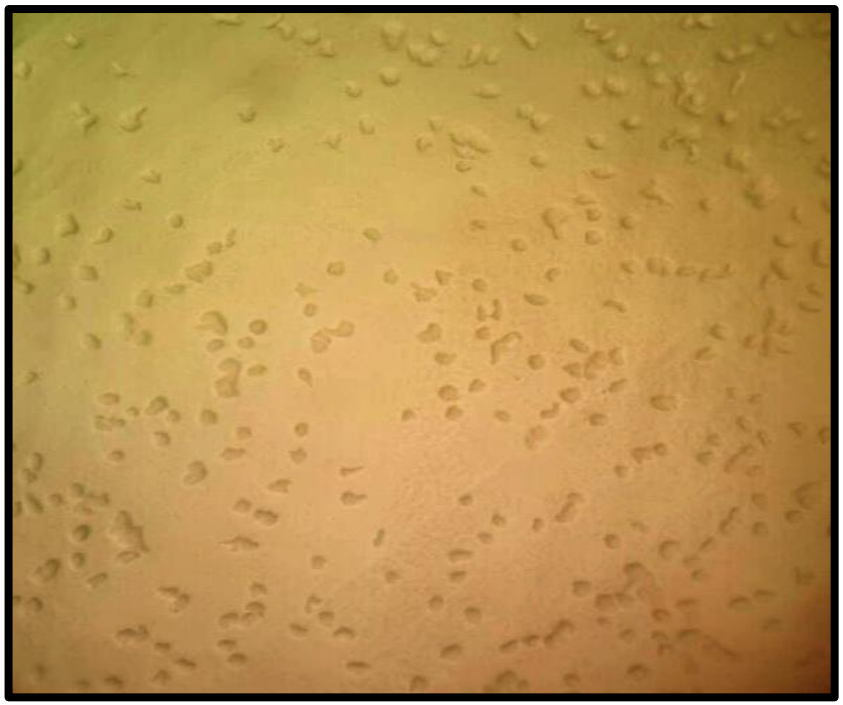




Pre Treatment Image



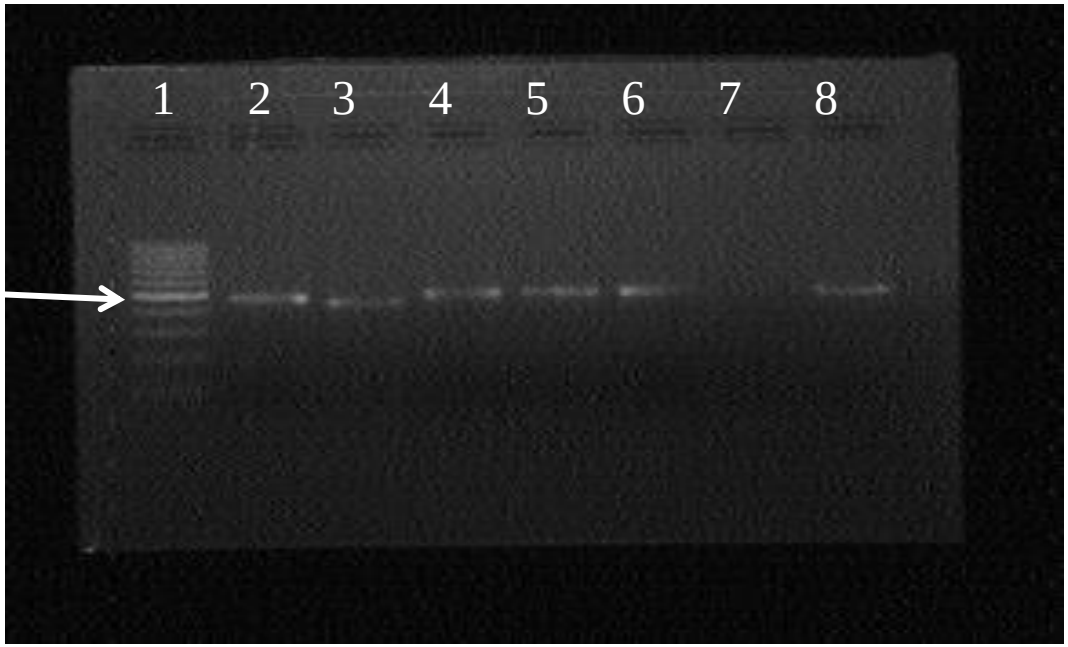
Post Treatment Image



Trophozoties

Cysts

Genus specific PCR amplification with JDP1 and JDP2



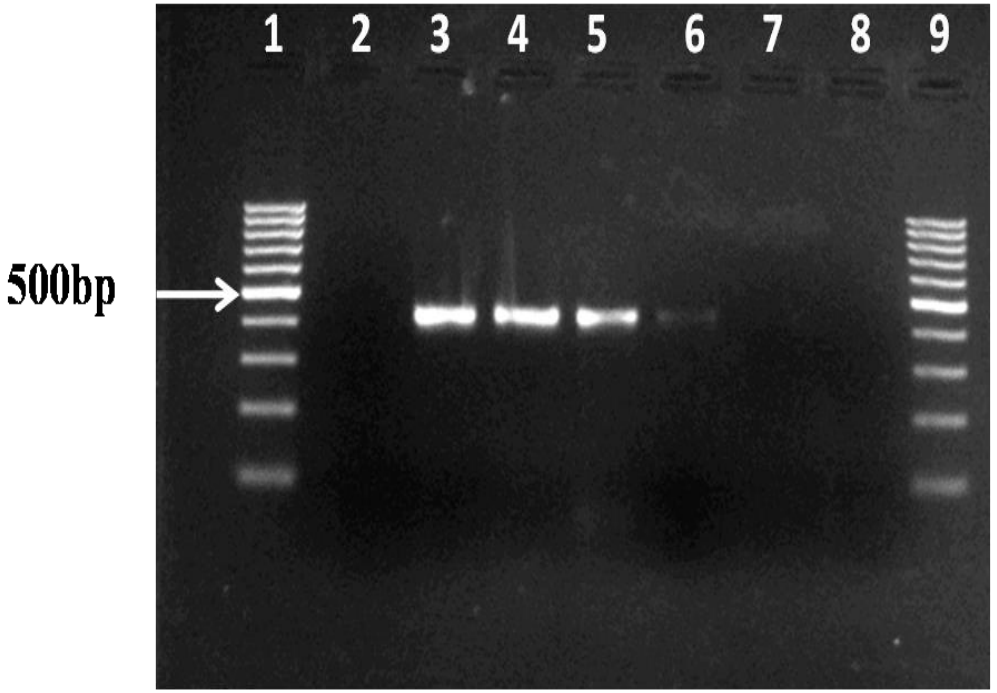
Lane: 1 – 100 bp Molecular marker

Lane: 2-6 - Corneal Samples positive for *Acanthamoeba*

Lane: 7 – Positive control (Laboratory Maintained isolates of *Acanthamoeba*)

Lane 8 - Negative Control (only milliQ water)

Analytical Sensitivity of PCR



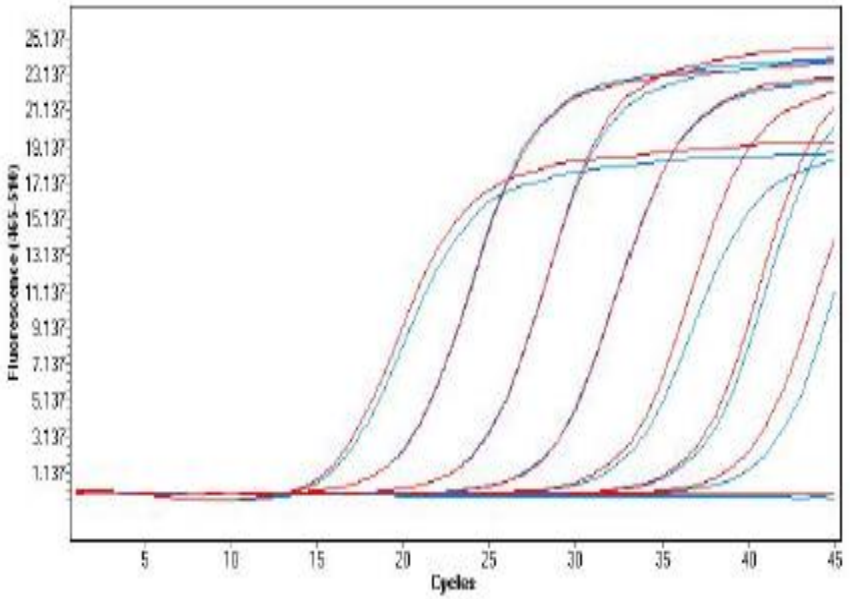
Lane :1and 9 - 100 bp Molecular marker

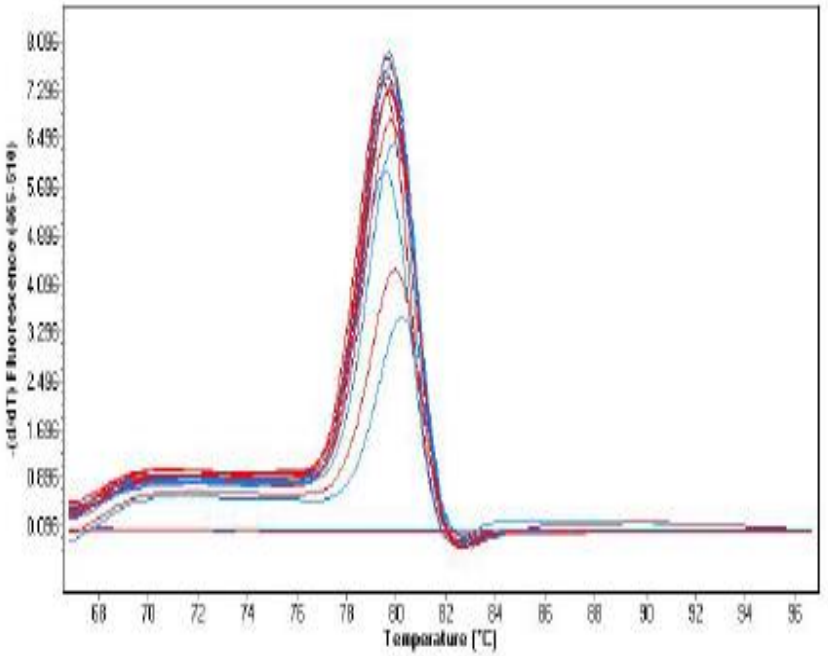
Lane: 2 - Negative Control (only milliQ water)

Lane: 3 to 8- 1ng to 10fg DNA conc

Amplification of amplicon from 1 pg of DNA = Single *Acanthamoeba*

Analytical Sensitivity of Real time PCR /Lower Limit of detection





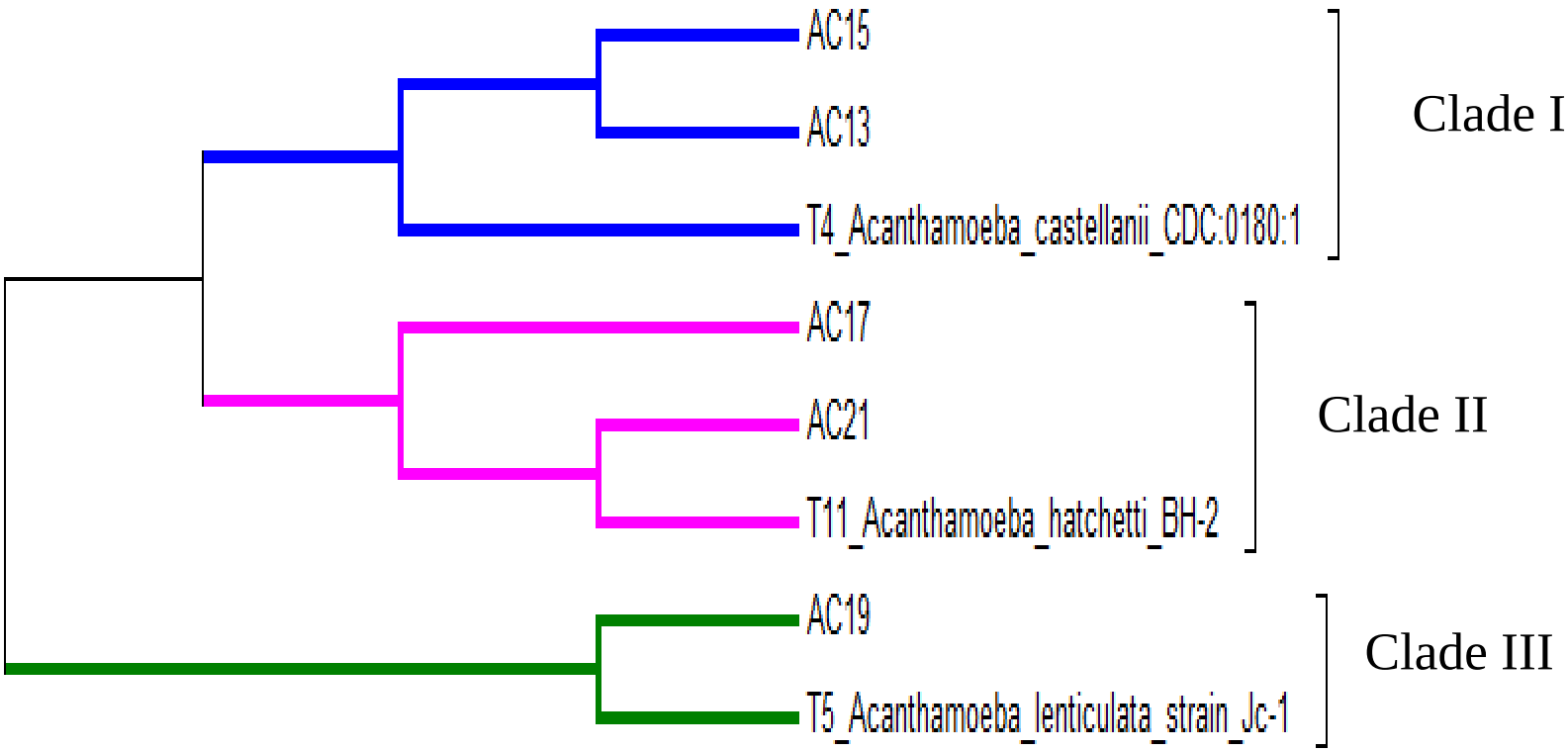
Amplification of amplicon from 100 fg of DNA = 1Copy of 18S rDNA

Sensitivity, specificity, positive predictive value , negative predictive value and accuracy of Microscopy, NNA culture, Polymerase chain reaction and Real time PCR for detection of *Acanthamoeba*

Test	Sensitivity	Specificity	PPV	NPV	Accuracy	Analytic sensitivity
Microscopy	20%	100%	100%	98.33%	98.33%	
NNA culture	100%	100%	100%	100%	100 %	
PCR	100%	100%	100%	100%	100%	1pg
Real -Time PCR	100%	100%	100%	100%	100%	100fg

Disease Prevalence 2.08%

Phylogenetic tree based on partial 18S rDNA sequences



Acanthamoeba Keratitis

T4-2- Trauma

T11-2 –Contact lens

T5-1

Discussion

References	Country	Findings	Genotype
Booton et al,2002	Hong kong	20 isolates	15-T4 5-T3
Zhang., et al, 2004	North China	26 isolates	25- T4 1-T3
Paul et al,2003	USA	62 Isolates	58- T4 2 -T3 2-T11
Ledee et al.,2009	USA	14 isolates	13- T4 1- T5
Sharma et al., 2004	India (PCR) (South India)	14 Isolates (Non contact lens wearer)	T4
Prashanth, et al., 2011	India (FAFLP) (South India)	15 isolate	T4 Five distinct clusters (I to V) within T 4 clonal complex
Our Study	**India**	**5 isolates**	**T4-2** **T5-1** **T11-2**

Conclusion

- Genotyping identification obtained in this study of *Acanthamoeba* isolated from AK confirmed T4 as the predominant genotype

- No study from North India regarding *Acanthamoeba* Genotyping from AK

- First study from India which reported T5 and T11 Genotype from AK

Acknowledgments

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Thank you