5th Diagnosis and Therapy of Fungal Diseases

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Molecular Characterization, Virulence Determinants and Antifungal Susceptibility Pattern of *Trichosporon asahii* isolates from new born babies in Nepal

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Introduction

- *Trichosporon asahii* is an emerging yeast causing deep seated infections in immunocompromised patients and in those on indwelling medical devices
- Mortality due to invasive Trichosporonosis is high, especially in the neonates and infants
- There are increasing reports of Trichosporonosis in the last one decade both in the adults and in the paediatric patients
- **Recent global epidemiological data highlight**
  - Emerging biofilm forming yeasts showing drug resistance
  - Genetic diversity among the strains with impact on their pathogenic attributes, drug resistance patterns, and on case management

OBJECTIVES

- To identify the prevalent genotypes of *Trichosporon asahii* isolates from Nepal
- To demonstrate their potential *in-vitro* virulence factors such as biofilm formation and other phenotypic enzymatic properties
- To evaluate the *in-vitro* antifungal susceptibility of the isolates under both planktonic and biofilm growth conditions

Materials and Methods

- Thirty one *T. asahii* from the following clinical samples were included in the study

  -Blood

  -Urine

  -Respiratory secretions /Endotracheal tubes

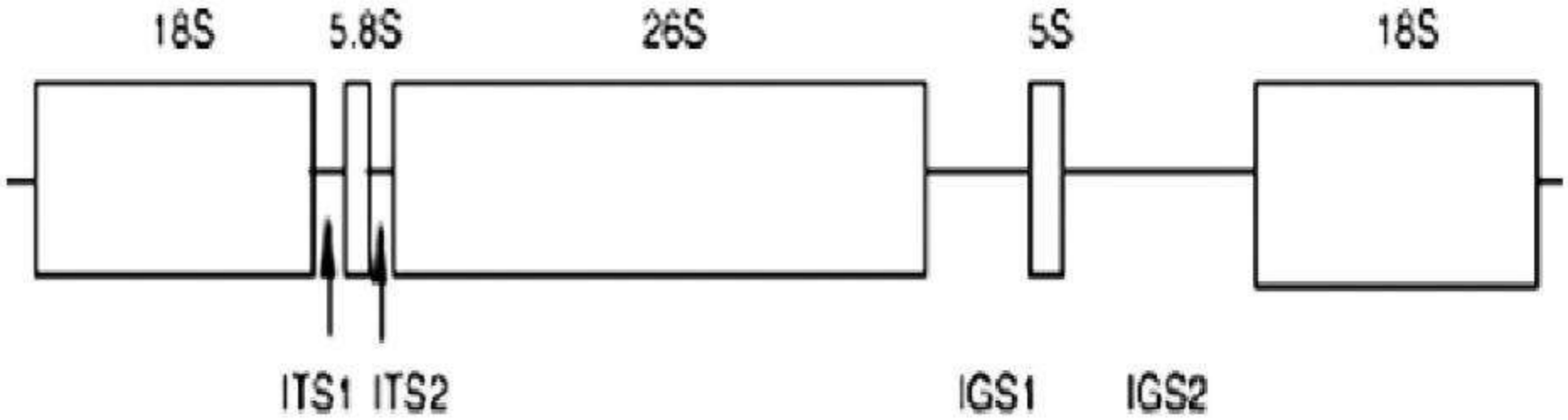
  -Pus

  -Indwelling devices (peripheral venous catheters,peritoneal dialysis catheter, urinary catheter)

  -Pleural fluid and peritoneal fluid

- Identified by conventional techniques and confirmed by amplification and sequencing of the IGS 1 region of the r DNA gene

r RNA gene of *Trichosporon*



Materials and Methods

- Molecular Identification

  -Genomic DNA obtained by Phenol chloroform extraction (Lee & Taylor 1990)

  -IGS 1 locus amplified using primers

  -26 s F (5’-ATCCTTTGCAGACGACTTGA-3’)

  -5s R (5’-AGCTTGACTTCGCAGATCGG 3’)

  -Amplicon purified by gel extraction kit (Quigen,Bengaluru,India)

  -Sequencing - Big Dye Terminator Cycle sequencing (Applied Biosystems, CA),analysed on ABI 3130 Genetic Analyzer (Applied Biosystems)

  -Sequences compared with available CBC-KNAW Fungal Biodiversity Center database and GeneBank DNA database

Materials and Methods

- Phenotypic Characterization of the isolates

  -Biofilm formation on microtiter plates

  (Measurement of Biofilm Biomass & Metabolic activity)

  -Enzymatic and other virulence properties

  -Proteinase

  -Phospholipase

  -DNase

  -Esterase

  -Superoxide dismutase

  -Haemolytic activity

  -Cell surface hydrophobicity

  -Melanin production

Materials and Methods

- ***In vitro* Antifungal Drug Sensitivity Assay**
  - -Planktonic cells – Broth microdilution technique (CLSI-M27-A3)
  - -Biofilm cells – XTT Reduction Assay in microtiter wells
- **Antifungal agents tested**
  - -Amphotericin B
  - -Fluconazole
  - -Voriconazole
  - -Itraconazole
  - -Posaconazole
  - -Caspofungin
  - -Anidulafungin
  - -Micafungin

RESULTS

Number of organisms isolated from various clinical samples

Nature of sample	No of isolates (* Isolates from neonates)
Indwelling medical devices	11 (3 from peripheral vascualr catheters *)
Blood	6 (3 neonatal septicemia *)
Respiratory secretions	3
Endotracheal tubes	2*
Urine	5
Peritoneal fluid	1
Pleural fluid Pus Total	2 1 * 31

Results

- Majority of the patients in this study were immunocompromised, being at extremes of ages, associated with comorbidities, and admitted to intensive care units and on prolonged broad-spectrum antibiotics.
- All 31 clinical isolates belonged to species *Trichosporon asahii.*
- Six of 31 isolates were genotype IV and rest 25 isolates were of genotype III.

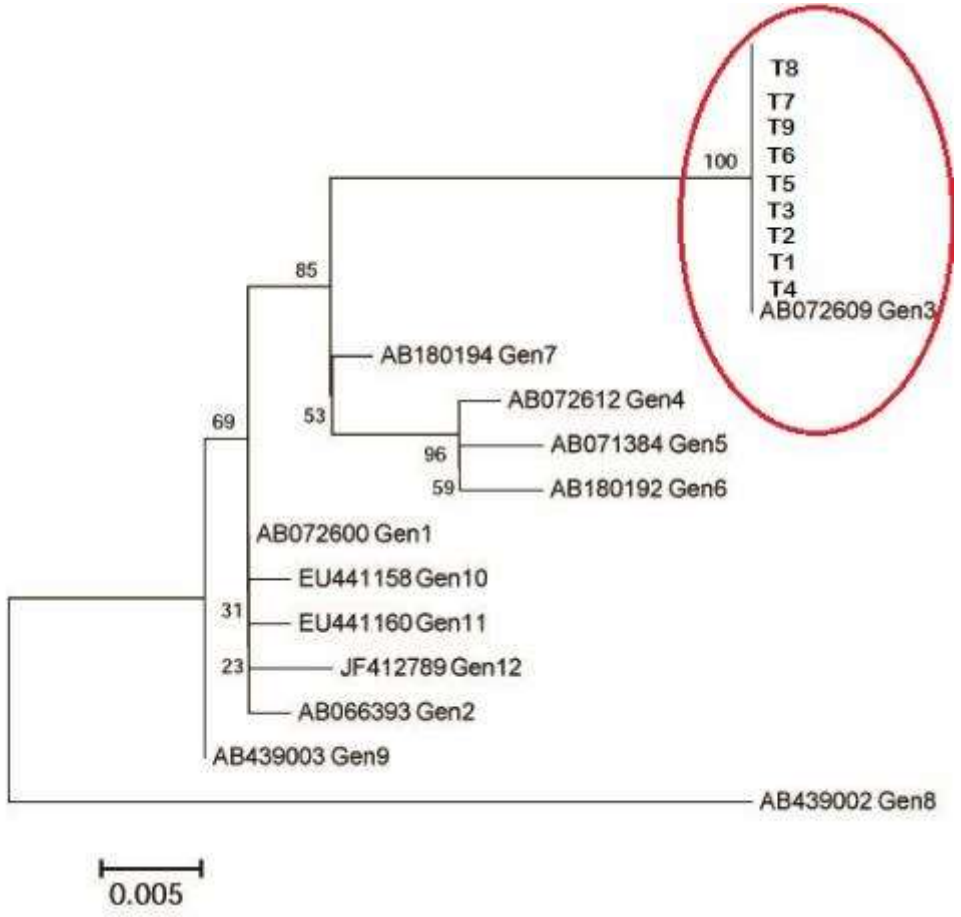
Clinical profile of nine cases of invasive *Trichosporon asahii* infection in neonates

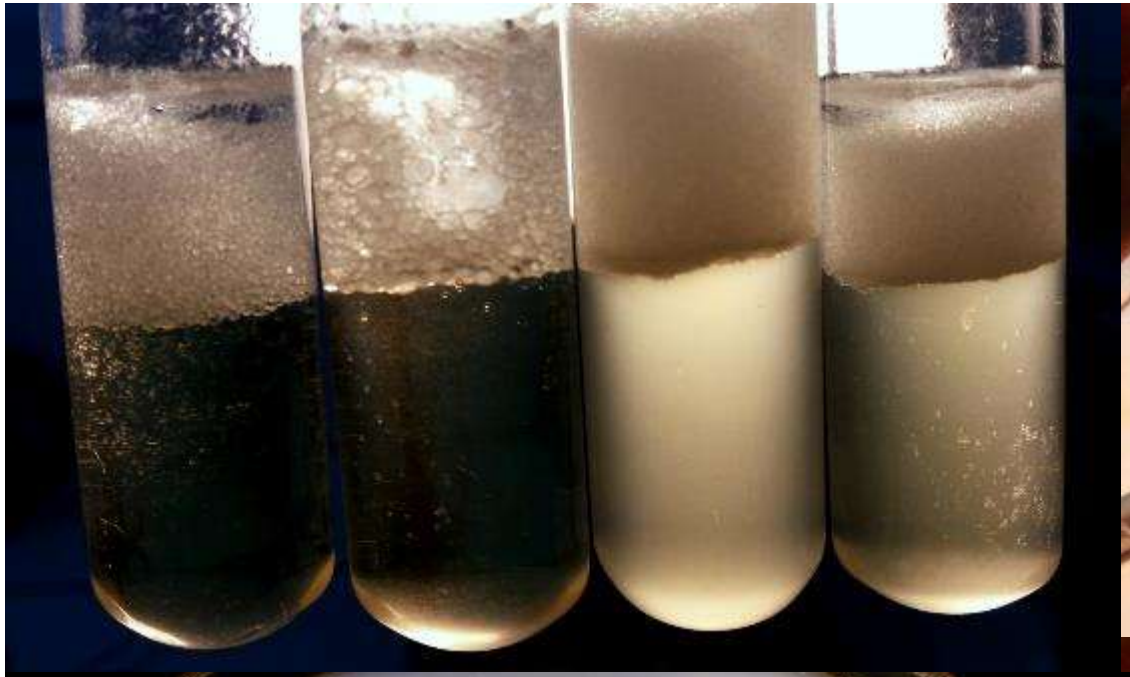
Case No.	1	2	3	4	5	6	7	8	9
Age/gender	New born/F	New born/F	7 days/F	10 days/M	28 days/F	11 days/M	5 days/M	22 days/M	8 days/M
Dept/Ward	NICU	NICU	NICU	NICU	Paed ICU	NICU	NICU	Paed ICU	NICU
Underlyig disease	Preterm LBW, with respiratory distress,apnoea,tracheostomy done	Neonatal sepsis	Neonatal sepsis	Preterm LBW ,Fever with convulsions, peripheral venous catheter	Respiratory distress, tracheostomy	Neonatal sepsis	Fever with peripheral venous catheter	Cellulitis left lower leg	Fever with peripheral venous catheter
Clinical specimen	Tracheostomy tube	Blood	Blood	IV catheter	Tracheostomy tube	Blood	IV catheter	Pus	IV catheter
Prior use of Antibiotics	yes	yes	yes	yes	yes	yes	yes	yes	yes

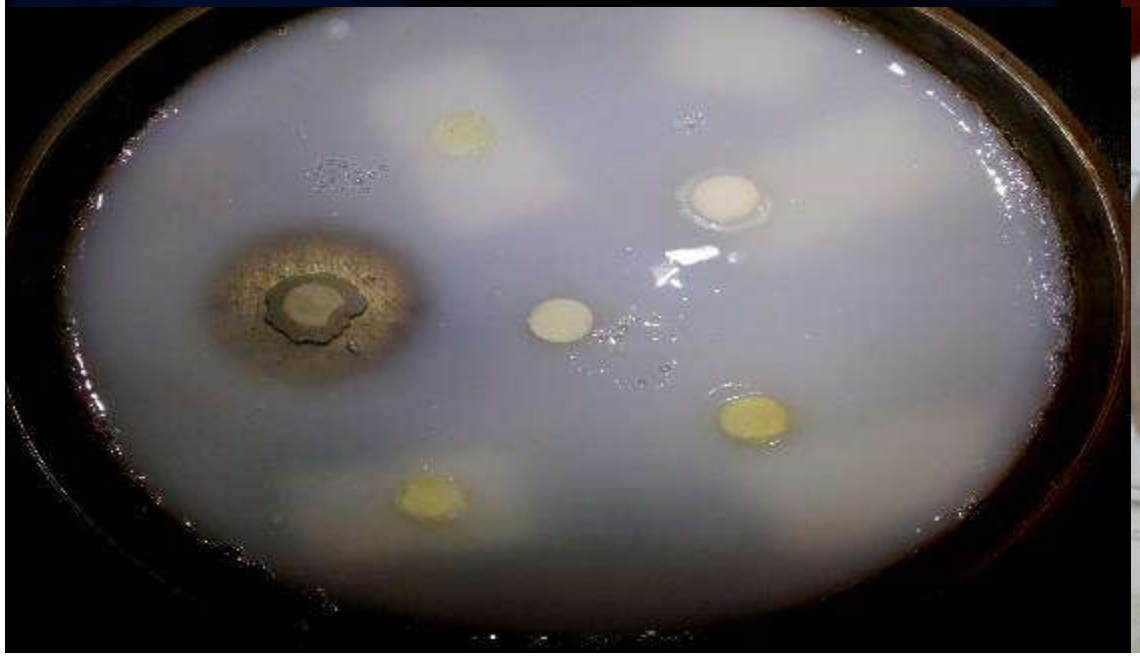
Clinical profile of nine cases of invasive *Trichosporon asahii* infection in neonates

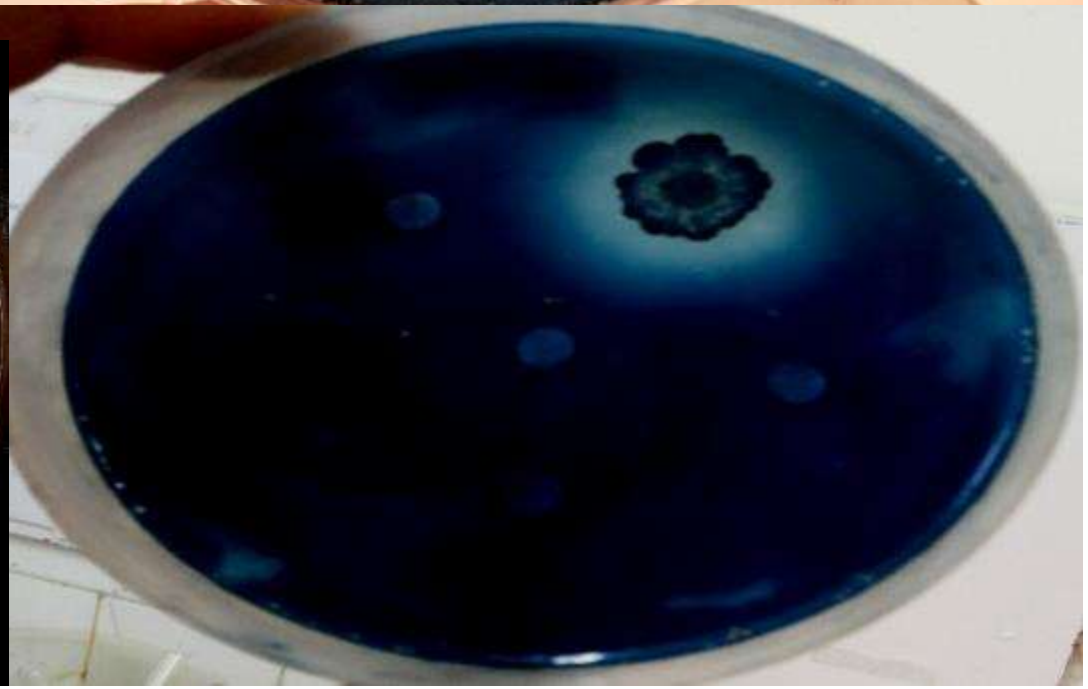
Case No.	1	2	3	4	5	6	7	8	9
Antifungal therapy	Fluconazole	Amp B	Amp B	Amp B +Fluconazole	Amp B	Fluconazole	AmpB + Fluconazole	Amp B	Amp B
Day of isolation and initiation of antifungal of therapy	5 days	3 days	5 days	4 days	6 days	6 days	5 days	NA	4 days
Outcome (cause of death)	Satisfactory clinical outcome	Satisfactory clinical out come	Satisfactory clinical outcome	Satisfactory clinical outcome	Died on the 8th day of admission respiratory arrest	Satisfactory clinical outcome	Satisfactory clinical outcome	Satisfactory clinical outcome	Satisfactory clinical out come

T asahii genotypes in neonates

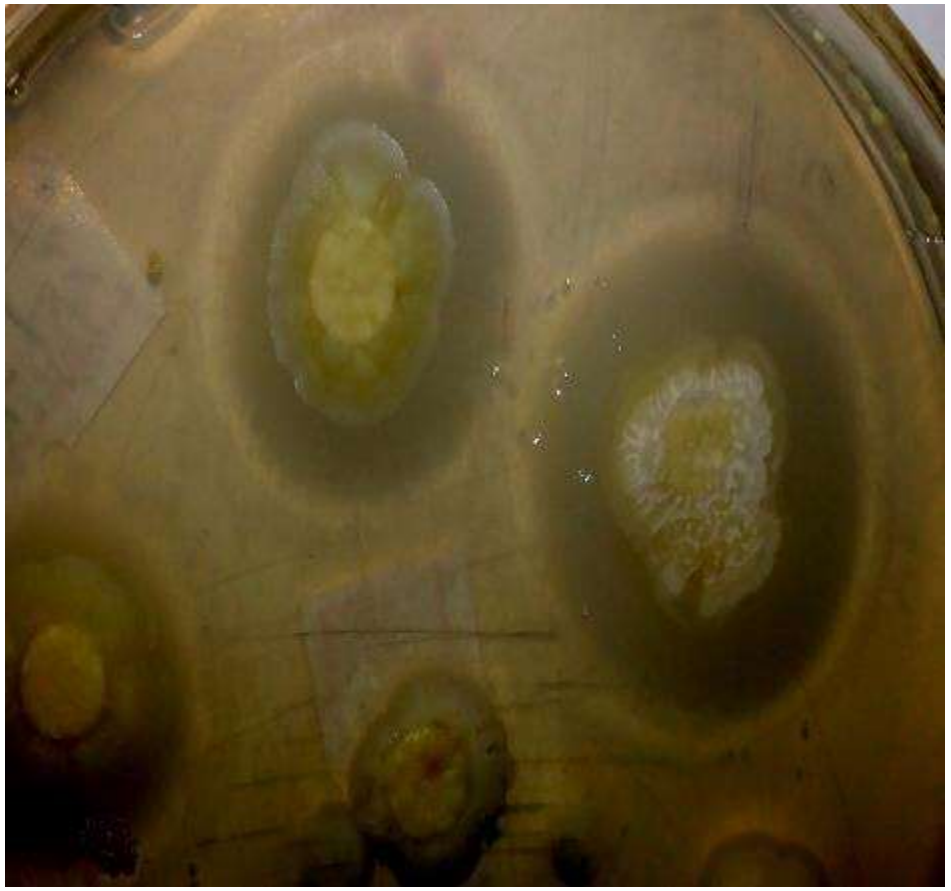


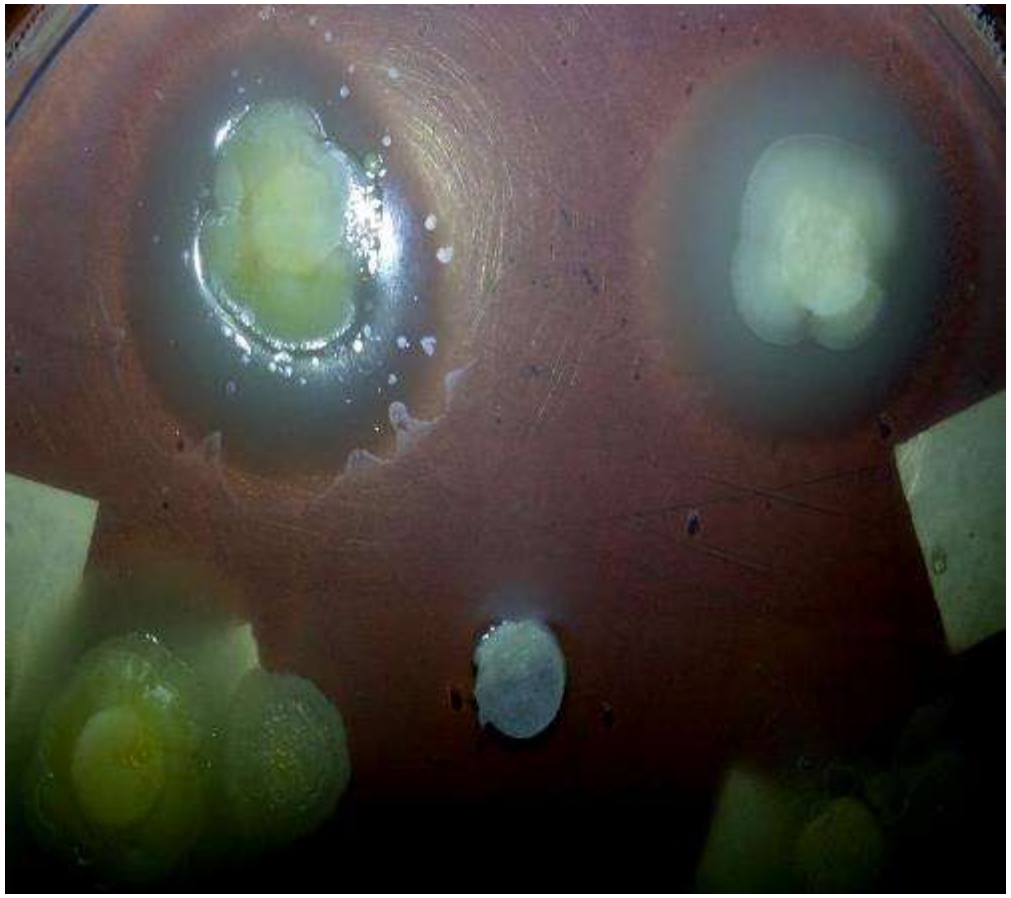






Esterase Test





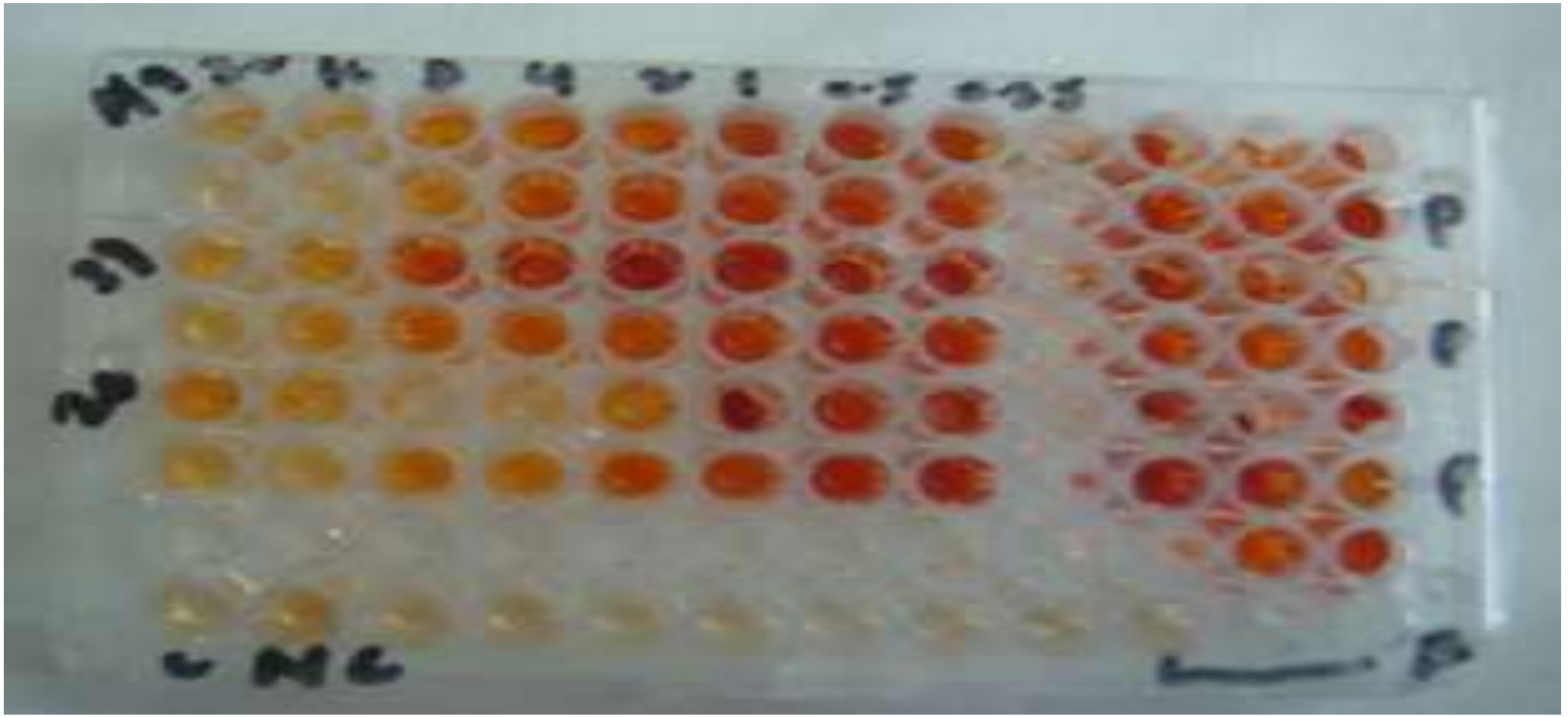
Extracellular enzymic activities and other phenotypic virulence markers of *T asahii* isolates (n=31)

Phenotypic property	No positive (%)	No Negative(%)
DNase	28 (90.3)	03 (9.7)
Phospholipase	15 (48.4)	16 (51.6)
Cell surface hydrophobicity (10-74%)	31 (100)	00(0)
Esterase	21 (67.7)	10 (32.3)
Haemolysin	00 (0)	31 (100)
Proteinase	07 (22.6)	24 (77.4)
Superoxide dismutase **5 Mm** **10mM** **15mM** **Melanin** **(L – Dopa)**	 27 (87.1) 10 (32.3) 03 (9.7) 31 (100)	 04 (12.9) 21 (67.7) 28 (90.3) 00 (0)

Semiquantitative Biofilm Assay Results of 31 *T asahii* isolates (measurement of metabolic activity and biomass)

Biofilm Assay Method	Moderate to high biofilm activity	Low Biofilm Activity
XTT Reduction Assay (Metabolic activity)	30 (96.8)	01 (3.2)
Crystal violet assay (Biofilm Biomass)	26 (83.9)	05 (16.1)

XTT (2,3-Bis-(2-Methoxy-4-Nitro 5- Sulfophenyl)-2H –Tetrazolium-5-Carboxaniliode)reduction assay



In vitro activities of antifungal agents against *T asahii* under planktonic and biofilm growth conditions

Antifungal agent	MIC 50 (ug/ml)planktonic	MIC 90 (ug/ml)planktonic	GMT planktonic	MIC 50 Biofilm	MIC90 Biofilm	GMT Biofilm
Amphotericin B	2	2	1.74	≥20	≥20	64
Fluconazole	8	8	6	≥800	≥800	1024
Voriconazole	0.125	0.49	0.18	≥12.5	≥49	64
Itraconazole	0.125	0.42	0.16	-	-	-
Posaconazole	0.3	0.42	0.25	-	-	-
Caspofungin	0.5	1	0.66	-	-	-
Anidulkafungin	0.5	0.8	0.37	-	-	-
Micafungin	4	8	5.27	-	-	-

CONCLUSIONS

- Genotype III was prevalent among the invasive *Trichosporon* isolates in Western Nepal
- Biofilm formation by *T asahii* was found as a major virulence trait that was related to the ability of the organism to grow on indwelling medical devices
- Biofilm cells showed higher MICs against fluconazole, amphotericin B, and voriconazole as compared to the planktonic cells
- Fluconazole and amphotericicn B were less active than itraconazole and voriconazole with higher geometric mean MIC

CONCLUSIONS

- Voriconazole and itraconazole *in vitro* were the most effective against planktonic *T asahii* isolates (geometric mean being 0.18 ug/ml and 0.16 ug/ml respectively)
- Due to poor physiological condition of severely ill patients in ICUs, a potent antifungal agent with much wider antifungal activity spectrum, such as voriconazole, may be recommended.
- Prompt species identification along with local antifungal susceptibility data may play a crucial role in patient management; though high rate of biofilm formation showing drug resistance remains a concern

Acknowledgements

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Thank You ☺
जनिपाल शिक्षण अस्पताल