DETERMINING CLINICAL RELEVANCE OF FUNGAL ISOLATES

APHL Webinar
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P.A.C.E. 588.918.13
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Faculty Disclosure

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Eszter Deak
“Nothing to disclose”
Objectives

- Discuss the ubiquity of fungi as a source of infection and contamination
- Define pathogenic and non-pathogenic (accidental, normal flora) fungi
- Review laboratory methods for diagnosis of fungi
- List the potential reasons for a positive fungal laboratory result
- Describe approaches to outbreak investigation, assessment of laboratory contamination versus hospital contamination
- Present cases
Those Ubiquitous Fungi

• Fungi can be found in soil, on plants, trees, and other vegetation, and in water
• Can be helpful – penicillin, bread, wine, and beer use ingredients made from fungi
• Commensals on our skin, respiratory, genital and intestinal tracts
• Exposure of an individual to an organism can lead to one of three outcomes. The organism can:
  o transiently colonize the individual
  o permanently colonize the individual
  o produce disease
Residential and Transient Fungi

Skin
- *Malassezia* spp
- *Candida* spp

Eyes
- *Penicillium* spp
- *Aspergillus* spp
- *Candida* spp
- *Rhodotorula*
- *Alternaria*
- *Cladosporium*

Oropharyngeal/Resp Tract
- *Candida* spp
- *Pneumocystis*
- *Aspergillus* spp
- *Penicillium* spp
- Filamentous basidiomycetes
- *Cryptococcus*
- *Rhodotorula* spp
- *Trichosporon* spp
- *Scedosporium prolificans*

GI Tract
- *Candida* spp

Genital Tract
- *Candida* spp
- *Saccharomyces* spp
“The closer you look, the more you find”

- Susan M. Huse of the Marine Biological Laboratory in Woods Hole, Mass., a contributor to the microbiome project.
The Fungus Among Us – Skin

- Study by National Human Genome Research Institute and the National Cancer Institute:
  - DNA sequencing techniques
  - 10 healthy adults
    - No chronic health conditions, dermatologic diseases or use of antimicrobials
  - Skin at 14 body sites
    - Predilection for skin disorders

Findley et al Nature 2013; doi:10.1038/nature12171
The Fungus Among Us – Skin

- Skin is home to two fungal phyla: Ascomycetes and Basidiomycetes
  - Arm sites: 18-32 fungal genera
  - Head and trunk: 2 to 10 fungal types
  - 80, 60 and 40 types of fungi on the heel, toenail and toe webbing, respectively
  - Genus *Malassezia* of the Basidiomycetes dominates at 11 core-body and arm sites.
    - 11 of 14 species represented
    - Species specificity at sites
  - *Malassezia* present on toenails, heels and toe webbing, but these sites showed much greater fungal diversity than rest of the body.
    - Person-to-person variability
Findley et al Nature 2013; doi:10.1038/nature12171

<table>
<thead>
<tr>
<th>Genera</th>
<th>Number of isolates cultured across skin sites in all HVs</th>
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<tbody>
<tr>
<td>Alternaria</td>
<td>1</td>
</tr>
<tr>
<td>Aspergillus</td>
<td>19</td>
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<tr>
<td>Candida</td>
<td>1</td>
</tr>
<tr>
<td>Chaetomium</td>
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</tr>
<tr>
<td>Chrysosporium</td>
<td>3</td>
</tr>
<tr>
<td>Cladosporium</td>
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<tr>
<td>Coprinellus</td>
<td>2</td>
</tr>
<tr>
<td>Malassezia</td>
<td>62</td>
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<tr>
<td>Mucor</td>
<td>1</td>
</tr>
<tr>
<td>Nigrospora</td>
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</tr>
<tr>
<td>Penicillium</td>
<td>25</td>
</tr>
<tr>
<td>Pestalotiopsis</td>
<td>2</td>
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<tr>
<td>Rhodotorula</td>
<td>5</td>
</tr>
<tr>
<td>Saccharomyces</td>
<td>1</td>
</tr>
<tr>
<td>Sclerotagontospora</td>
<td>1</td>
</tr>
<tr>
<td>Scolecobasidium</td>
<td>1</td>
</tr>
<tr>
<td>Thielavia</td>
<td>1</td>
</tr>
<tr>
<td>Trichophyton</td>
<td>4</td>
</tr>
</tbody>
</table>

Table S2. List of cultured genera from culture-based approach.
Genera isolated from the initial sampling of skin sites are listed with number of individual isolates cultured.
The Fungus Among Us – Oral

• Oral fungal microbiome study at Case Western
  o Used multitag pyrosequencing
  o Concentrated oral rinse from 20 healthy individuals

• Overall, oral cavity contained 74 culturable and 11 non-culturable fungal genera

Ghannoum et al Plos Path 2010; 6(1):e1000713
The Fungus Among Us – Oral

- 9-23 species ID’d from oral rinse of study participants: *Candida* (75%), *Cladosporium* (65%), *Aureobasidium* (50%), *Saccharomycetales* (50%), *Aspergillus* (35%), *Fusarium* (30%) and *Cryptococcus* (20%).

- Of the *Candida* spp, 40% were *C. albicans*, *C. parapsilosis* (15%), *C. tropicalis* (15%), *C. metapsilosis* (5%), and *C. khmerensis* (5%)

- Shifting oral microbiome with HIV infection
A Fungus Among Us – GI Tract

- Study looking at feces of 23 mice
- 30 Mb of raw data from 454 pyrosequencing and over 2.2 GB of raw data from Illumina GA sequencing together containing over 1.3 million individual sequences
- Overall saw significant amounts of fungal DNA

Iliev et al Science 2012; 336:1314-7
A Fungus Among Us – GI Tract

- Detailed analysis identified over 100 different well-annotated fungal species representing at least 50 genera
- Identified over 100 novel/unannotated fungi
- 97.3% of all the fungal sequences identified belonged to 10 fungal species with 65.2% of the sequences belonging to a single fungus: *Candida tropicalis*
Epidemiology of Fungal Infections

- Expansion of immunocompromised patient population has led to an increased incidence of invasive fungal infections.

- The Transplant-Associated Infections Surveillance Network (TRANSNET)
  - Consortium of 23 transplant centers in the United States
  - Prospective study of the epidemiology of IFIs in solid-organ transplant (SOT) and Hematopoietic Stem Cell Transplant Recipients (HSCT) recipients over a 5-year period from 2001 to 2006.

Changing Epidemiology of Fungal Infections

• Increasing frequency of non-albicans Candida spp in invasive candidiasis

A. n = 1198

Changing Epidemiology of Fungal Infections

- In HSCT recipients, *Aspergillus* species are predominant
  - Invasive aspergillosis (43%) was more common than invasive candidiasis (28%)
  - Invasive aspergillosis in Intensive Care Unit (ICU) on the rise
  - Mortality rates, 70-90% in HSCT
  - Emergence of new opportunistic pathogens, including non-*fumigatus* *Aspergillus* spp

Changing Epidemiology of Fungal Infections

- Hyaline molds (*Fusarium* species, *Acremonium*, *Paecilomyces*, *Scedosporium*, and *Trichoderma*, the dematiaceous fungi (*Alternaria*, *Bipolaris*, *Curvularia*, *Cladosporium*, and *Exserohilum* species), and the agents of mucormycosis increasing
  - Voriconazole prophylaxis use

![Pie chart showing the frequency of different fungal infections](chart.png)
Epidemiology of Fungal Infections

- *Candida* spp still most common cause of invasive fungal infection
  - 4th most common pathogen in BSI
- The most common invasive fungal infections in the SOT recipients were candidiasis (53% of all invasive fungal infections found) followed by invasive aspergillosis (19%), cryptococcosis (8%), non-Aspergillus molds (8%), endemic fungi (5%), and mucormycosis (2%).

Changing Epidemiology of Fungal Infections

- The incidence of infections caused by *Cryptococcus neoformans* has risen markedly over the last 20 years.
- Non-neoformans cryptococci regarded as saprophytes, rarely reported as human pathogens:
  - Increased over the last few decades
  - *Cryptococcus albidus* responsible for 80% of reported cases

Khawcharoenporn et al. *Infection* 2007; 35:51-58
Changing Epidemiology of Fungal Infections

- Recently, invasive *C. gattii* infections in immunocompetent hosts have been reported in Western Canada, mainly Vancouver Island and the North West region of the USA.
  - The organism, which is thought to thrive only in tropical regions, has been recovered in some temperate climate zone countries.
Fungal Identification

- Yeasts
  - KOH/Calcofluor Wet Mount
  - Culture
    - Germ tube/Urease
    - Chromagar
    - Cornmeal Agar
    - PNA FISH
    - Vitek
    - API Strips
    - Maldi-Tof
    - Sequencing
Fungal Identification

- **Molds**
  - Serology for antigen
  - Culture (SAB, PFA)
    - Colony morphology
    - Microscopic appearance
      - Tape Prep
      - Tease Mount
  - Maldi-Tof
  - Sequencing
Fungal Identification

- **Cryptococcus spp**
  - Budding yeast with no hyphae or pseudohyphae
  - Urease Positive
  - Bird Seed Agar
    - Produces brown melanin-like pigment
    - Can have bird seed negative isolates
  - Cryptococcal antigen detection (IMMY LFA)
  - Maldi-Tof
  - Sequencing
Fungal Identification

- *C. gattii* vs *C. neoformans*
- L-Canavanine glycine bromo-thymol blue
  - *C. gattii* assimilates glycine (blue)
- Clinical Presentation of *C. gattii*
  - Affects immunocompetent and immunocompromised individuals
  - Elicits muted host response?
  - Multiple lesions in lungs and brain
  - Disseminated skin and lung lesions resemble malignancies
  - Slower to respond to therapy

<table>
<thead>
<tr>
<th>Species</th>
<th>Serotype</th>
<th>Molecular Type</th>
<th>Distribution (more prevalent regions)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. neoformans</em></td>
<td>A</td>
<td>VNI</td>
<td>Global (Australia, Africa, North America)</td>
</tr>
<tr>
<td><em>C. neoformans</em></td>
<td>B</td>
<td>VGI</td>
<td>Global (Australia)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VGII</td>
<td>North America (Pacific Northwest) &amp; South America (Columbia)</td>
</tr>
<tr>
<td><em>C. gattii</em></td>
<td>C</td>
<td>VGIII</td>
<td>South America</td>
</tr>
<tr>
<td><em>C. gattii</em></td>
<td>D</td>
<td>VNIIV</td>
<td>North America</td>
</tr>
<tr>
<td><em>C. neoformans</em></td>
<td>A/D</td>
<td>VNIII</td>
<td>Global (Europe)</td>
</tr>
<tr>
<td><em>C. neoformans</em></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

IMMY LFA Domestic White Paper
Positive Fungal Lab Results

• True Infection
  o Exogenous source
    • Hospital acquired infection
      – Person-to-person
      – Dirty instruments
      – Contaminated water
      – Inhalation
      – Injection of contaminated drugs, steroids, etc
  • Community acquired
    – Contaminated foreign body penetrates skin (tornado)
  • Multiple individuals affected by same/closely related strain
Positive Fungal Lab Results

• True Infection
  o Endogenous source
    • Normal Flora
      – Catheter
      – Trache
    • Change in immune status/normal flora
      – Antibiotics
      – Hormones
      – Steroids
  • Isolated events
Positive Fungal Lab Results

• Contamination
  o Exogenous source
    • Contaminated specimen collection instruments
    • Laboratory contamination
    • Multiple individuals/specimens affected by same/closely related strains
  o Endogenous source
    • Normal Flora
Laboratory Diagnostic Challenges

- Widespread in nature, often cultured from diseased body surfaces, may be difficult to assess whether a fungus found during disease is actually a pathogen.

- Interpretation of results of fungal cultures may be difficult due to frequent colonization of certain body sites.

- Many of environmental organisms that are etiologic agents of opportunistic mycoses also involved in contamination of specimens, cultures.
Laboratory Diagnostic Challenges

• Before a specific fungus can be confirmed as cause of disease, the same fungus must be isolated from serial specimens and fungal elements morphologically consistent with the isolate must be observed in tissues taken from lesion.

• Most isolates of *Candida* spp, *C. neoformans*, and *Fusarium* spp. obtained from blood cultures are clinically significant.
Laboratory Diagnostic Challenges

• *Aspergillus* spp and *Penicillium* spp can represent pseudofungemia or contamination.

• Isolation of *Aspergillus* spp. from cultures of the respiratory tract specimens may not be clinically significant because it is found in the environment and can colonize the airways of individuals without causing overt disease.

Laboratory Diagnostic Challenges

• Isolation of *Candida* in urine in pure cultures may or may not suggest that it is causing a UTI, even in high numbers
  o Need to look at the patient clinically
  o Asymptomatic lower genital tract carriage of *Candida* spp. is estimated to occur in 21-32% of healthy women, with *C. albicans* representing 20-98% of identified isolates
  Sobel Lancet 2007; 369:1961-71
Laboratory Diagnostic Challenges

- *Candida* rarely causes pneumonia
  - Candida colonization is extensive throughout the respiratory tree in some critically ill patients undergoing mechanical ventilation
  - Candida is a normal inhabitant of the mouth, and can be recovered from sputum in 20 to 55% of normal subjects
  - Diagnostic performances of PSB, endotracheal aspirates, BAL, blind lung biopsies, and bronchoscopically guided lung biopsies in one study were not optimal
    - The use of quantitative cultures of Candida in respiratory samples is not helpful for establishing this type of infection
  - Need to correlate culture with histopathology

Barenfanger et al JCM 2003; 41:5645-9;
El-Ebiary et al Am J Respir Crit Care Med 1997;156:583-590
Outbreak Setting

• Is it a true infection or pseudo outbreak
  o Is the clinical presentation in the patients consistent with the laboratory diagnosis
  o Index of suspicion

• Is there contamination in the laboratory
  o Is there more than one organism isolated from each of the patients
  o Types of specimens isolated from – make sense?
  o Is there a pattern as far as where the samples are coming from?
Outbreak Settings

• Establish relatedness of isolates
  o Genus
  o Species
  o Strain
    • Molecular methods for subtyping
      – Multilocus Sequence Typing (MLST)
        » Sequencing and alignment of multiple loci
      – Amplified Fragment Length Polymorphism (AFLP)
        » Digestion of total cellular DNA with one or more restriction enzymes and looking at fragments
      – Variable Number Tandem Repeats
        » A microsatellite/minisatellite consists of a specific sequence of DNA bases or nucleotides which contains tandem repeats
          • AAAAAAAAAA would be referred to as (A)_11
          • GTGTGTGTGTGT would be referred to as (GT)_6
          • CTGCTGCTGCTG would be referred to as (CTG)_4
          • ACTCACTCACTCCTC would be referred to as (ACTC)_4
        » widely dispersed in eukaryotic genomes, are highly variable
      – Inter Simple Sequence Repeats (ISSR)
Variable Number Tandem Repeats (VNTR)

5’ ATCGGTCGTTGATGTAACGA 3’

Examples:
TATATATATA = (TA)$_5$ dinucleotide
CAGCAGCAG = (CAG)$_3$ trinucleotide
VNTRs – why?

• Based on the premise that variable numbers of these repeated sequences arise due to DNA polymerase errors during replication or meiosis
• High rate of mutation
• Allows for identification of strains between species
VNTRs

**Strengths**
- Specific primers repeatable results
- Reliable automated fragment sizing

**Weaknesses**
- Single locus analysis
- Need to have a sequenced genome
- Some debate as to the stability of VNTR
Inter Simple Sequence Repeat (ISSR)
ISSR

**Strengths**
- Long primers high annealing temperatures repeatable results
- Compares different parts of the genome
- No prior knowledge of genome sequence
- Reliable automated fragment sizing

**Weakness**
- only works where simple sequence repeats exist in a specific pattern
AFLP - amplified fragment length polymorphism

1. DNA is “chopped up” using specific restriction enzymes
2. Adaptors of known sequence are ligated to ends of pieces
3. Linkers serve as templates for primers
4. DNA is amplified and run on gel/sized
5. Different strains/species will have different profiles
Fungal Sub-typing tools for Outbreak Investigation

• Methods generate isolate-specific molecular fingerprints for assessment of epidemiological relatedness.
• Typing data should be considered within the time frame and the current epidemiological context
• Focus is on identifying sources of outbreak
Assumptions

- Isolates associated with the OB are a recent progeny of a single ancestor
- Such isolates will have the same genotype
- Epidemiologically unrelated isolates will have different genotypes
**Outbreak Settings**

- Determine of source of organism
  - Commonalities between patients
    - Hospital, clinic, floor, room
    - Proximity of room
    - Equipment flow
    - Healthcare worker
    - Procedure
    - Lot numbers of drugs if used
    - Construction (molds)
    - Recent Cleaning activities (molds and vents)
    - Collection of specimen
    - Processing or aliquoting of specimen
    - Laboratory testing
  - Environmental sampling
Challenges

• Fungi are ubiquitous
• Presence of organism doesn’t necessarily correlate with disease
• Source of infection no longer present
• Subtyping results can be ambiguous
Case Study 1

- 44 year old nonsmoking female schoolteacher from Bihar, India
- Persistent productive cough, on-and-off hemoptysis for 8 yrs; progressive breathlessness for 4 yrs, right-sided chest pain and high-grade fever for 15 days
- History of Raynaud’s phenomenon and received anti tuberculosis treatment (ATT) several times over past 7 yrs
- Chest radiograph: cavitating consolidation, infiltrates and pleural effusion
- Repeated sputum cultures grew Nocardia
- Put on iv imipenem and cotrimoxazole

Chowdhary et al JCM 2013; 51:585-90
Case Study 1

- Systems persisted and pleural effusion increased
- KOH wet mount of sputum showed presence of hyaline, septate, and branched hyphae; multiple colonies of white mold isolated from sputum cultures: *Ceriporia lacerata*
- Confirmed growth of Mtb
- Started on isoniazid, rifampin, pyrazinimide, and ethambutol and im streptomycin plus cotrimoxazole
- 3 month follow-up: no growth of *Nocardia* or Mtb in culture; *C. lacerata* still grew
- Discontinued streptomycin and pyrazinimide
Case Study 1

• Continued to improve

• Stopped ATT, maintained on cotrimoxazole
  Continues to improve without antifungals

• Case of asymptomatic colonization of respiratory tract by basidiomycete mold, no further therapeutic intervention undertaken
Case Study 2

- 34-year-old man diagnosed with acute myeloid leukaemia
- He responded favourably to remission-induction chemotherapy followed by two courses of intensive consolidation
- Underwent an uncomplicated allogeneic peripheral blood stem cell transplantation from an HLA-identical sibling but relapsed
- Underwent reinduction chemotherapy but had complications, transferred to ICU
- New episode of neutropenic fever while receiving meropenem therapy empirically. Blood cultures positive for *Staphylococcus epidermidis*
- Daily radiographs of the chest showed bilateral pulmonary infiltrates that remained unchanged
- Patient defervesced after the initiation of vancomycin
Case Study 2

- Diagnosis of probable invasive aspergillosis made based on a progressive increase in serum galactomannan antigenaemia, progression of pulmonary infiltrates and development of new consolidations on chest X-ray, dyspnoea and cough, and microscopic demonstration (Grocott staining) of moulds compatible with Aspergillus species in a BAL sample.
- Cultures taken from two consecutive BAL samples yielded ‘fungi’
- Started on caspofungin
- Clinical condition continued to deteriorate. A high-resolution CT scan of the lungs demonstrated multiple large nodules in both lungs, surrounded by a halo of lower attenuation.
- The patient passed shortly thereafter.

Lagrou et al JMM 2005; 54:685-88
Case Study 2

- Fungus isolated from BAL grew within 2 days of incubation at 37 °C on a chocolate agar plate and after 8 days (2 days at 37 °C, followed by 6 days at 22 °C) on Sabouraud glucose (2 %) agar containing chloramphenicol.
- Macroscopically, multiple white- to slightly cream-coloured dense colonies were observed. Production of conidia could be stimulated by subcultivation on Takashio medium (Sabouraud agar with 0.2 % glucose).
- Microscopic examination of the fungus revealed conidiophores from which clusters of rectangular arthroconidia were produced. The arthroconidia were cylindrical with truncate ends but with terminal cells rounded at the tip.
- No fruit bodies were obtained in culture.
- These morphological characteristics led to a tentative identification of the fungus as *Hormographiella* species, later ID’d as *Coprinus cinereus* (anamorph *Hormographiella aspergillata*) by amplification and sequencing of the internal transcribed spacer 2 (ITS2) region.
Case Study 3

• Possible *Aspergillus fumigatus* contamination problem in an off-site laboratory following duct cleaning

• Concomittant with this, ICU was in the middle of an outbreak of *A. fumigatus* among patients

• Hospital epidemiologist concerned ICU outbreak is a pseudo-outbreak from the lab

• Strain typing was performed to evaluate possibility of laboratory contamination and to determine whether the ICU outbreak could be linked to what was happening in the laboratory
• A microsatellite method was employed for strain typing
• There were a few predominant strains of *A. fumigatus* present
• Clustering of isolates from contaminated patient plates and air sampling plates was observed (boxed in green)
• These isolates were not clinically relevant
• ICU Strains were clearly different from the lab contamination isolates
Case Study 4

- *C. guilliermondii* was isolated from 64 of 149 (43%) blood cultures mainly obtained over a one month period from the Pediatric Emergency Department (Pediatric ED) of a Hospital.

- Patients had no previous exposure to risk factors for candidemia and did not present any clinical evidence of systemic infection.

- The microbiological investigation of materials and surfaces showed the presence of *C. guilliermondii* in the sphygmomanometer cuff, paper towels, and sink drain at the Pediatric ED.

- Analysis of the samples collected from the 32 members of the nursing staff showed that 20 (79%) were colonized with fungi, and that in 7 cases (21%), the colonization was due to *C. guilliermondii*.

Medeiros et al JCM 2007; 45:942-7
Case Study 4

• 25 isolates of *C. guilliermondii* from the hospital cluster were subtyped, 20 from blood cultures and isolates from three nurses' hands, and two environmental sources related to the cluster (sphygmomanometer cuff and paper towel). Non-outbreak isolates were added for controls.

• Random amplification of polymorphic DNA was used as subtyping method
  o It is a type of PCR reaction, but the segments of DNA that are amplified are random
  o Several arbitrary, short primers (8–12 nucleotides) are used for PCR of a large template of genomic DNA, hoping that fragments will amplify
  o By resolving the resulting patterns on a gel, a semi-unique profile can be gleaned from a RAPD reaction.

• For seven of the eight RAPD primers, no reproducible differences were observed among the 25 isolates

• However, all primers gave different patterns for the nonoutbreak isolates
Case Study 4

- It was concluded from the subtyping results that the pseudo-outbreak strains from the patients are similar and are related to some of the environmental strains.

- This supports the hypothesis that this cluster originated from one or a limited number of common sources.

- Adoption of intervention measures was effective in resolving the outbreak, supporting the hypothesis that the outbreak was due to poor techniques of drawing blood samples for culture.
Summary

• Fungi are everywhere

• Changing epidemiology of fungi
  o Incidence
  o Severity
  o Patient populations

• Opportunistic infections can be found as part of resident or transient flora

• Environmental contaminants can cause disease

• Becoming more and more difficult to assess clinical relevance of fungal isolates
Summary

• With the continuous expanding spectrum of fungi causing invasive disease, it is important to consider the clinical relevance of every fungus cultured from samples of immunocompromised patients.
  o Contact between the microbiologist and clinician is therefore indispensable.

• Temperature study
  o If it does not grow at around 30°C, it’s probably not a pathogen

• Tissue samples showing invasion may be necessary to establish invasive infection

• In outbreak situations where the presence of the organism in culture is unknown to result from hospital/laboratory contamination or true infection, epidemiologic investigation and molecular typing can be performed to identify a source
THANK YOU