VIRULENCE FACTORS AND SUSCEPTIBILITY OF *CANDIDA* SPP. CAUSATIVE AGENTS OF NEONATAL INFECTIONS

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What do we know?

- Third most common microorganism isolated from obligatory sterile body sites

- High mortality in neonates, especially in those with low birth weight (LBW)

- Numerous risk factors: indwelling devices, broad-spectrum antibiotics, total parenteral nutrition, corticosteroids, gastrointestinal surgery, and/or history of fungal colonization (Chicella et al., 2011, J Pediatr Pharmacol Ther.).

- Empiric antifungal therapy results in improved survival

What we still need to learn?

-What are the most important virulence factors?

-Can we predict the disease progression based on virulence factors?

-Are the predisposing factors for disease development reliable?

AIMS:

-Determine *Candida* spp. from blood cultures in neonates with sepsis

-Evaluate their susceptibility

-Determine virulence characteristics

-Correlate the obtained data with collected patients' data

Materials and methods

-Patients' data collection from the Clinical Centre Niš, Department of neonatology included:

- -Age,
- -Gender,
- -Underlaying diseases,
- -Hospitalization related data,
- -Therapy applied,
- -Disease outcome.

Candida spp. determination

-Blood culture cultivation in Bactec Mycosis Media (BACTEC 9120 system),

-Culturing and interpretation of colored colony morphology (HiCrome Candida Differential Agar),

-Interpretation of biochemical tests for clinical yeast identification (Fungifast, ELITech),

-Matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry

Susceptibility determination

Antifungal susceptibility testing was performed using Micronaut-AM MHK-2 plates (Merlin Diagnostika, Bornheim, Germany) that included:

-amphotericin B (AmB) (0.03-16 μg/ml),
-fluconazole (FLUC) (0.125-128 μg/ml),
-5-fluorocytosine (5-FLU) (0.06-32 μg/ml),
-itraconazole (ITR) (0.03-4 μg/ml),
-posaconazole (POS) (0.015-16 μg/ml),
-voriconazole (VOR) (0.015-16 μg/ml),
-micafungin (MCF) (0.01-16 μg/ml),
-caspofungin (CAS) (0.01-16 μg/ml),
-anidulafungin (ADF) (0.01-16 μg/ml)

Biofilm formation under static conditions

-Incubation performed for 48 h at 37 °C in RPMI containing 8% glucose,

-Staining was performed with 0.4% (w/v) crystal violet solution,

-Biofilm mass was distained with 96% (v/v) ethanol,

-The ethanol solution absorbance was recorded at 595 nm,

-Production classification: non-, weak, moderate and strong production Stepanović et al., (2000).

Rat macrophages isolation and preparation

-Wistar rats, male, 3 month old, injected with thioglicolic acid to elicit macrophages into the peritoneal cavity

-The peritoneal lavages obtained from animals were centrifuged (1200 rpm, 10 min at 4 ºC)

-Cell viability was determined by the trypan blue dye exclusion method

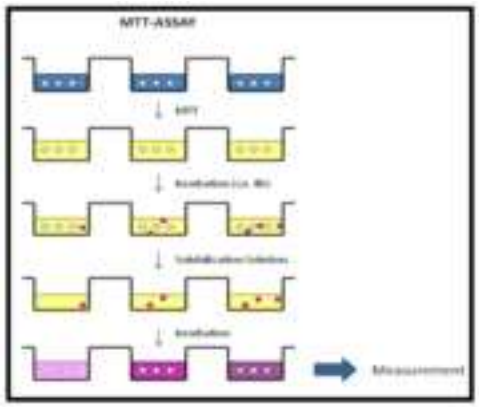
-Cell suspensions were adjusted to 2.5×10^6 cells/ml

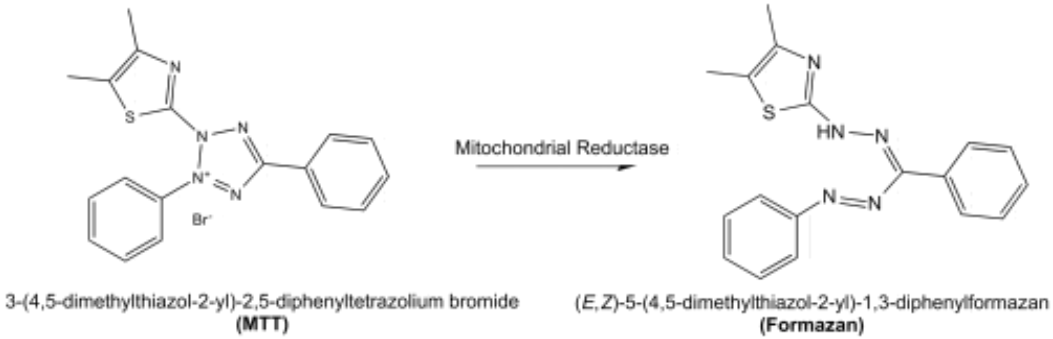
-All experiments were preformed according to Ethical regulations (EU Directive of 2010;2010/63/EU)

Macrophage viability and function

-Incubation of macrophages with *C. albicans* or *C. lusitaniae* for 24 h

-Determination of macrophage killing activity using MTT assay





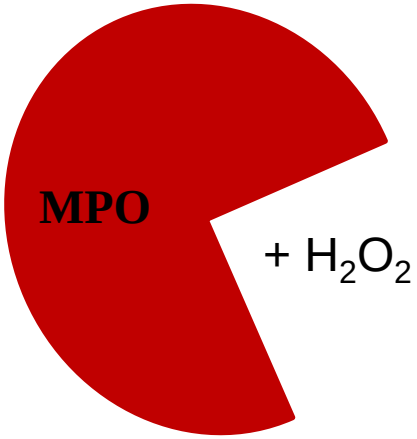
3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
(MTT)

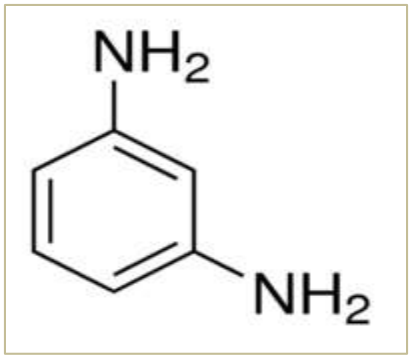
(*E*,*Z*)-5-(4,5-dimethylthiazol-2-yl)-1,3-diphenylformazan
(Formazan)

-Macrophage killing is expressed as % of viable macrophages after 24 h incubation with *C. albicans* or *C. lusitaniae*

Myeloperoxidase activty

-Incubation of macrophages with *C. albicans* or *C. lusitaniae* for 24 h





-MPO activity was presented as optical density (OD) increase compared to untreated cells x 1000

Candidacidal activity of macrophages

-Incubation of macrophages with *Candida* suspensions was preformed during 1 h

-After 24 h of incubation at 37 °C, viable counting of survivor cells (number of colonies) was performed.

-The results are expressed as hundreds of cells per ml.

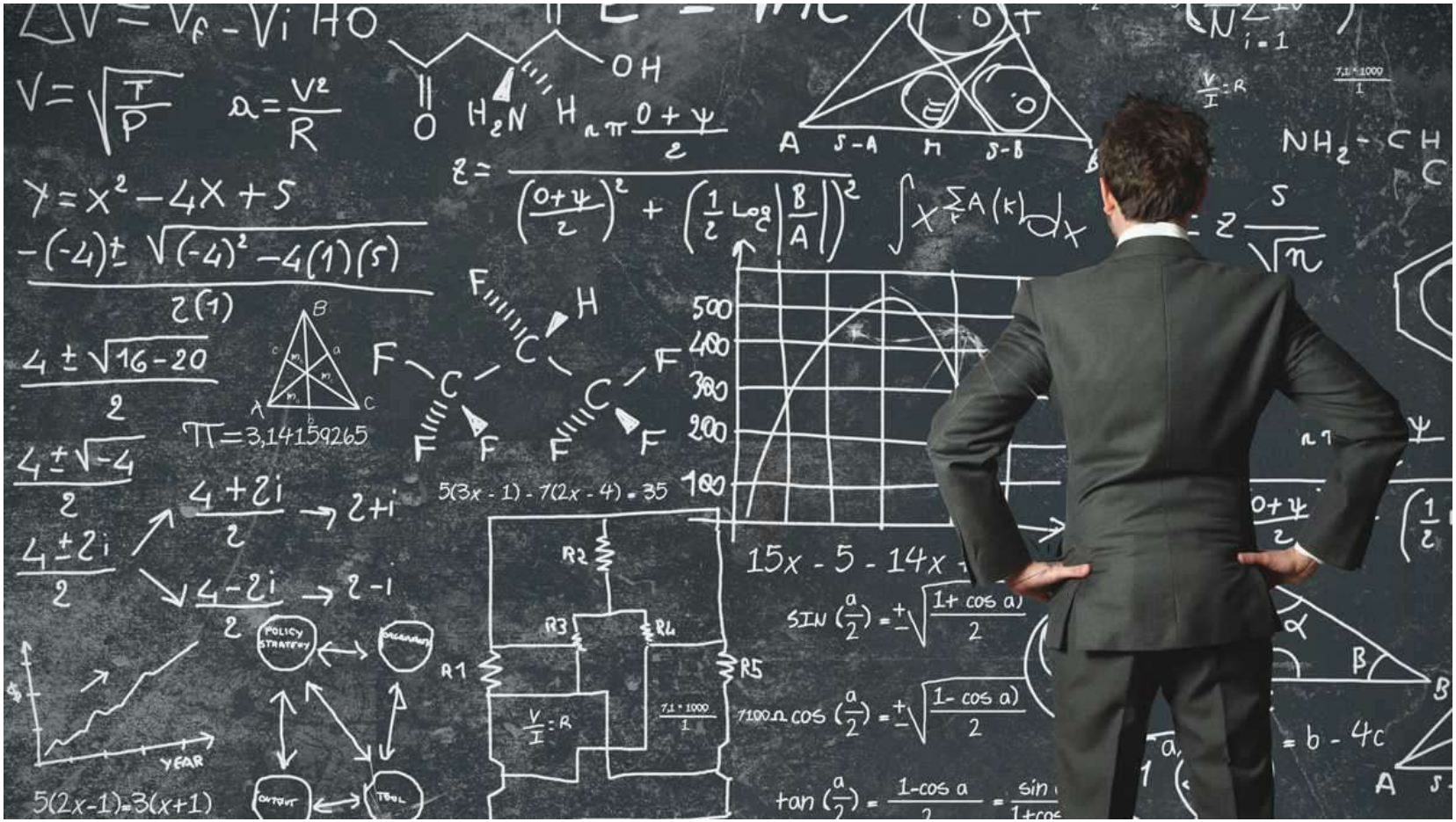
Phagocytosis assay

-Done on microscopic slides with previously adhered macrophages that were covered by *Candida* suspension (90 minutes at 25 °C) and stained with Giemsa

-The phagocytic index was determined by calculating the average number of yeast cells engulfed by or adhering to 100 macrophages.

-The percentage phagocytosis and the phagocytic index were calculated following the formula: percentage phagocytosis = number of positive cells per 100 cells observed x phagocytic index - average number of yeast cells engulfed by a positive cell.

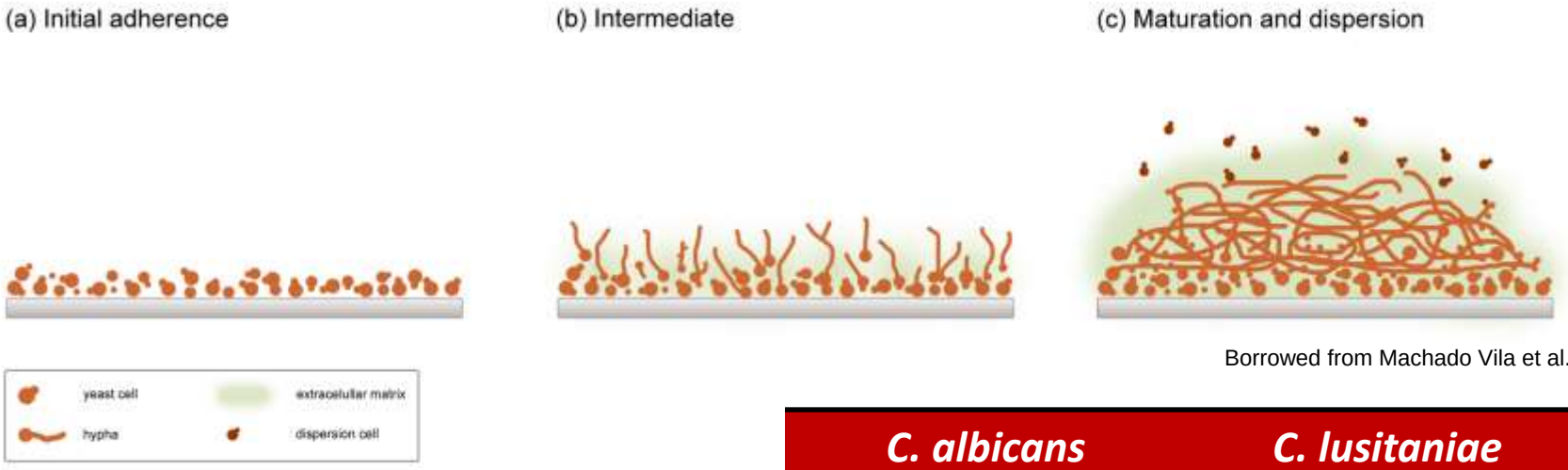
OUR FINDINGS



Patients' characteristics	No 1	No 2
Isolated strain	*C. albicans*	*C. lusitaniae*
Age (days)	17	15
Gender	M	M
Disease	LBW	LBW
Outcome	Cured	Cured

Strain/patients' characteristics	No 1	No 2
Isolated strain	*C. albicans*	*C. lusitaniae*
Resistance	None	None
Therapy applied	FLUC	AmB
Hyphal transition	+	-

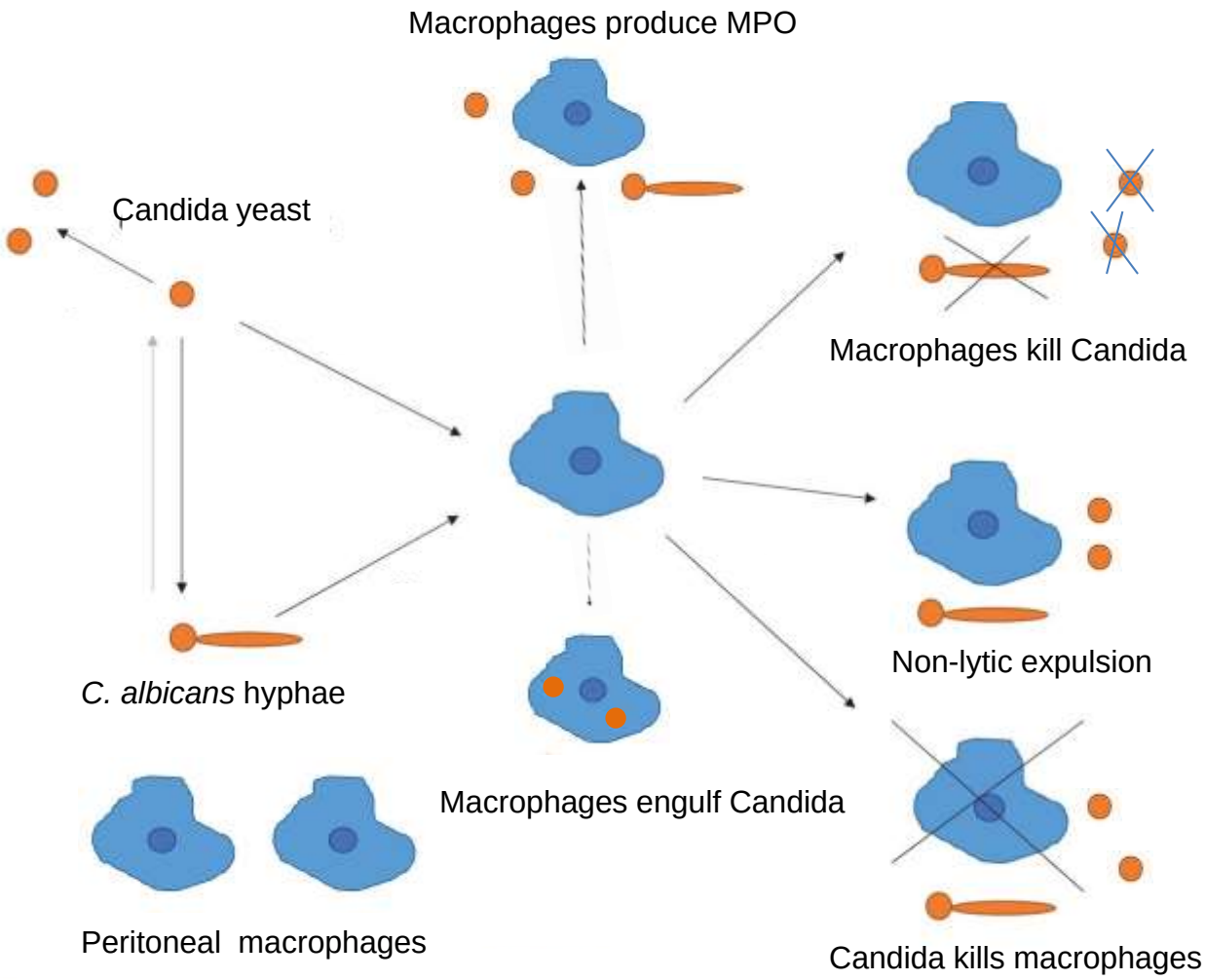
Biofilm production ability



Borrowed from Machado Vila et al., 2016

Risk factors: High blood glucose
Central venous catheter
Urinary catheter

C. albicans	*C. lusitaniae*
Strong	Weak
-	-
-	+


Macrophages produce MPO
Candida yeast
Macrophages kill Candida
Non-lytic expulsion
C. albicans hyphae
Macrophages engulf Candida
Peritoneal macrophages
Candida kills macrophages

Strain virulence characteristics	No 1	No 2
Isolated strain	*C. albicans*	*C. lusitaniae*
Macrophage killing activity (%)	78	76
MPO activity (ODx1000)	427	364
Candidacidal activity of macrophages (x 10^2 cells)	19	26
Macrophage phagocytosis assay (index)	5	27

Conclusions

-The results of the present study are only preliminary and although there are no differences, at least clearly visible ones, among two neonates the virulence characteristics of the two isolated strains exist.

-The authors are aware of the low number of strains that were evaluated and compared, but one cannot diminish the importance of the results obtained here.

-Obtained results indicate that NAC, in this case *C. lusitaniae*, is more sensitive to the influence of host cells, while *C. albicans* causes a more intense reaction of host cells

Thank you for your kind attention