ASSESSMENT OF THE IN VITRO ANTIFUNGIC EFFECT OF MXP-4509 ON YEAST STRAINS USING EUCAST DEF.7.1 STANDARDISED METHOD

MATERIALS AND METHODS

The used test procedure was following the standard of the European Committee of Antifungal Susceptibility Testing (EUCAST Def. 7.1). The test is performed in titre microplates, using decreasing binare dilutions of antifungic substance.

The recommended testing mediun is RPMI 1640 (with glutamine, a pH indicator and no bicarbonate), supplemented with glucose at a final concentration of 2%. The use of a higher glucose quantity leads to a better growth for yeasts and facilitates the readings for Minimal Inhibitory Concentrations. The use of MOPS (acid 3-(N-morpholino) propanesulfonic) with a final concentration of 0.165mol/L, mentains the optimal pH during incubation period.

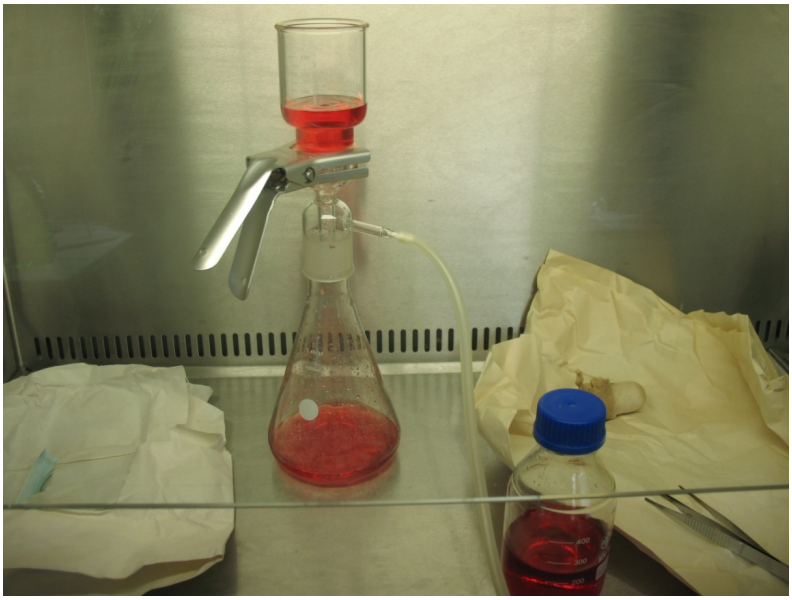
The RPMI medium with 2% glucose (Table 1) is prepared as described below:

1. The compounds from Table 1 in 600 ml distilated water
2. It is homogenised until complete disolvation of the compounds
3. During homogenization the pH is adjusted at value 7 and 25ºC, with a sodium hidroxyde 1N solution.
4. Water is added until it reaches the one litre volume.
5. Is sterilised using a Millipore filter 0.22µm.
6. Conservation of the substance is at 4º C.
7. For quality control tests is used a sterilised medium sample inoculated with a reference strain and a new determination of pH.

Table 1

Medium components RPMI 2% glucose

Components	**x 2 Concentration**
Distiled water	900mL
RPMI 1640	20.8g
MOPS	69.06g
Glucose	36g



Filtering sterilisation of the RPMI 1640 medium

Antifungal agents. Preparation of the stock solution.

Were comparatively tested three antifungal agents from triazole class: MXP-4509, voriconazole and fluconazole. The powders were conserved in sigilled containers at -20ºC.

The stock antifungal substances were prepared taking into account the potency of the group. The necessary powder quantity was calculated as described bellow:

$$\text{Powder quantity (g)} = \frac{\text{Volume (L) x Concentration (mg/L)}}{\text{Potency (mg/g)}}$$

$$\text{Volume (mg/L)} = \frac{\text{Weight (g) x Potency (mg/g)}}{\text{Concentration (mg/L)}}$$

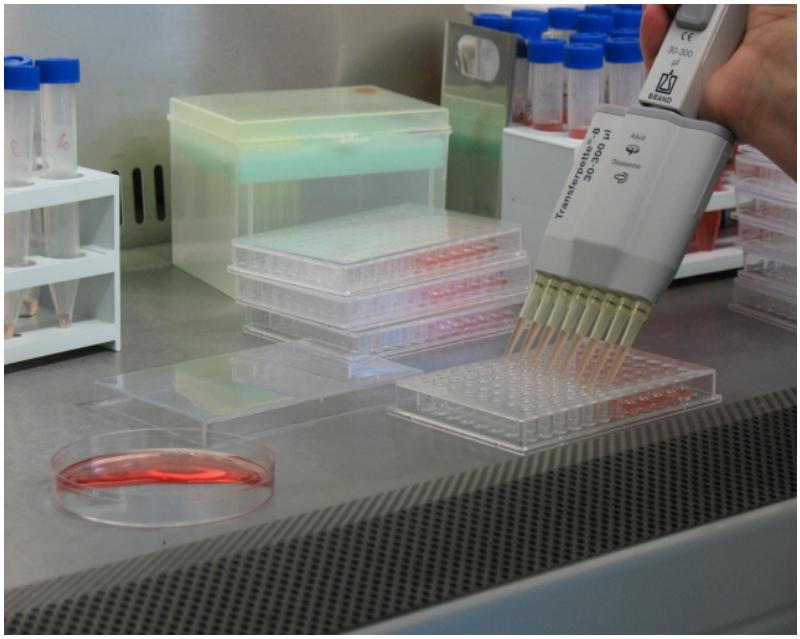
The antifungal powder is weightened using an analythical balance calibrated with two decimals. It is advisable to weighten minimally 100 mg. The powders were solubilised in sterile distilled water to a final concentration of 200 times higher than the final one.

Preparation of working substances

The variety of tested concentrations depends on the antifungal agent and must contain the breakpoint when is available, together with the published values for the control strains. The concentration values obtained in final after adition of the inoculum are: 64 – 0.125 mg/L for fluconazole, respectively 8 – 0.0156 mg/L for MXP-4509 and voriconazole.

The preparation of the microplates:

Were used sterile plastic plates, with 96 cupules and plate bottom with an aproximative capacity of 300 µL. In each row from 1 to 10 were distributed 100 µL from each tube containing the appropriate antifungic concentration. Thus, the rows 1-10 contained 100 µL antifungic substance in double concentrated medium with 1% solvent and rows 11 to 12 contained only double concentrated culture medium.



Distribution of the antifungical dilutions in the cupules of the microplate

Preparation of the inoculum

The standardisation of the inoculum is essential for susceptibility tests of antifungal agents. The final inoculum has to contain between $1x10^5$ and $2.5x10^5$ CFU/mL.

From the young culture on Sabouraud Agar, was prepared a suspension in sterile distilled water with a turbidity equivalent to 0.5 McFarland (approximately $2\text{-}5x10^6$ CFU/ml). It follows a second dilution in sterile distilled water of 1:10, to the final inoculum. Were tested a total of 273 yeast strains.

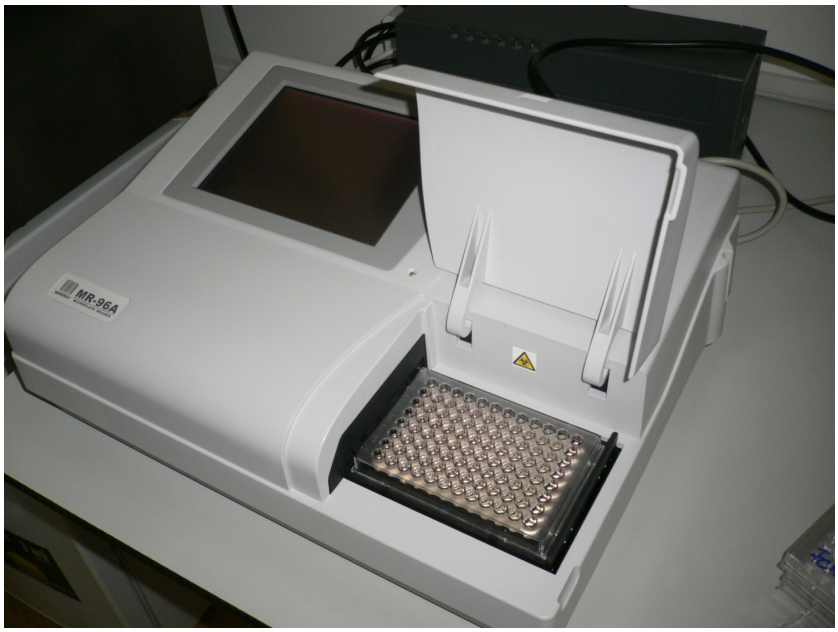
Inoculation of the microplates

It is advised for the inoculation to be performed in maximum 30 minutes from the standardisation of the suspension to mentain the viability of the yeast strains. Each cupule was inoculated with 100µL yeast suspension.

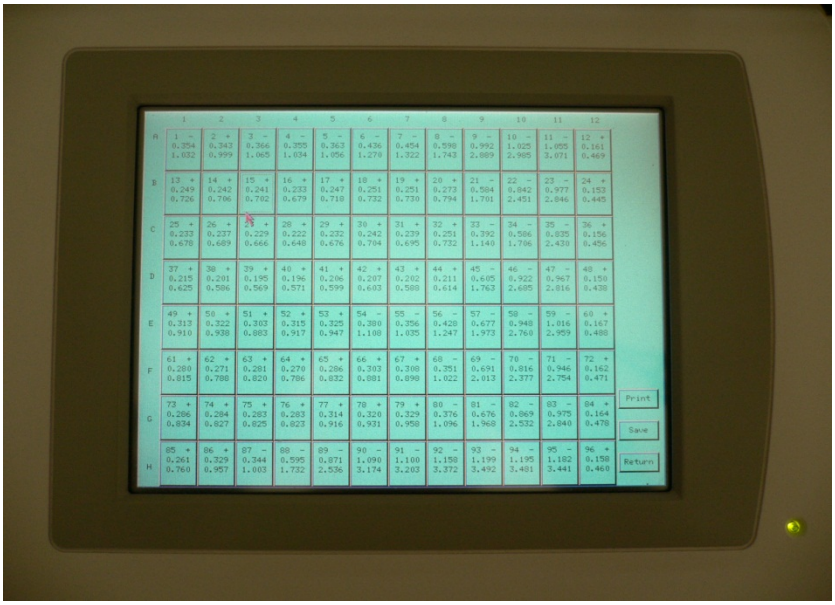
The incubation of the microplates was static, at 35-37ºC, for 24 hours. Exceptionally, it was necessary a new incubation period for 24 hours to obtain an adequate growth in control cupules, but a longer incubation period is not advisable.

Reading and interpretation of the results

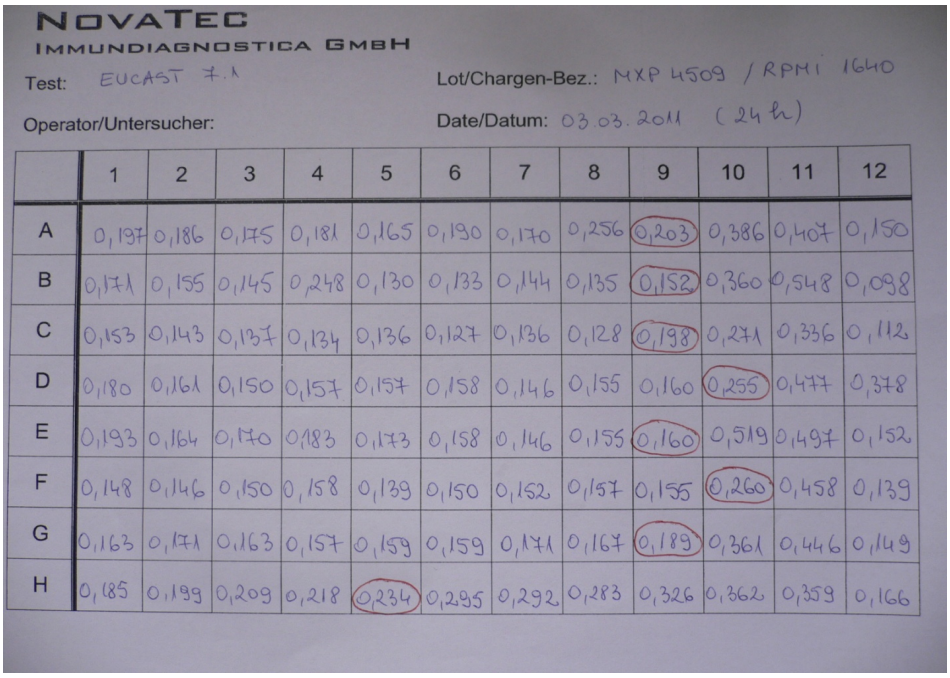
The final result was read with a MR-96 spectrofotometer (Mindray-China), marking down the absorbance of each cupule at 405 nm. The MICs for each azole antifungal agent is calculated as the lowest concentration that inhibits 50% the yeast growth (in comparison with the positive sample from cupule 11).



Spectrophotometer for microplates MR-96 (Mindray-China)



The display of the spectrophotometer with the results



NOVATEC
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Test: EUCAST 7.1 Lot/Chargen-Bez.: MXP 4509 / RPMI 1640

Operator/Untersucher: Date/Datum: 03.03.2011 (24 h)

	1	2	3	4	5	6	7	8	9	10	11	12
A	0,197	0,186	0,175	0,181	0,165	0,190	0,170	0,256	0,203	0,386	0,407	0,150
B	0,171	0,155	0,145	0,248	0,130	0,133	0,144	0,135	0,152	0,360	0,548	0,098
C	0,153	0,143	0,137	0,134	0,136	0,127	0,136	0,128	0,198	0,271	0,336	0,112
D	0,180	0,161	0,150	0,157	0,157	0,158	0,146	0,155	0,160	0,255	0,477	0,378
E	0,193	0,164	0,170	0,183	0,173	0,158	0,146	0,155	0,160	0,519	0,497	0,152
F	0,148	0,146	0,150	0,158	0,139	0,150	0,152	0,157	0,155	0,260	0,458	0,139
G	0,163	0,171	0,163	0,157	0,159	0,159	0,171	0,167	0,189	0,361	0,446	0,149
H	0,185	0,199	0,209	0,218	0,234	0,295	0,292	0,283	0,326	0,362	0,359	0,166

The determination of MIC in registration data sheets

Results

The synthesis of results was presented in Table 2.

Table 2

The antifungical effect of three azole agents – data synthesis

Antifungic agent	Concentration range (μg/ml)	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)
Fluconazole	>64-0.125	0.5	32
Voriconazole	>8-0.0156	0.0156	0.25
MXP 4509	>8-0.0156	0.0312	0.25

MIC – minimal inhibitory concentration
(50 – for 50% of strains, 90 – for 90% of strains)

The detailed results of the tests are isllustrated in Table 3. The distribution of the minimal inhibitory concentrations of MXP-4509 was similar to that of voriconazole (MIC_{50}: 0.0312 mg/L versus 0.0156 mg/L; MIC_{90}: 0.25 mg/L versus 0.25 mg/L), but significantly different to that of fluconazole (MIC_{50}: 0.0312 mg/L versus 0.5 mg/L; MIC_{90}: 0.25 mg/L versus 32 mg/L). The new MXP-4509 triazole proves a good antifungal activity *in vitro* on strains belonging to different genera and species that raise the interest towards future tests for its *in vivo* efficacy.

Table 3

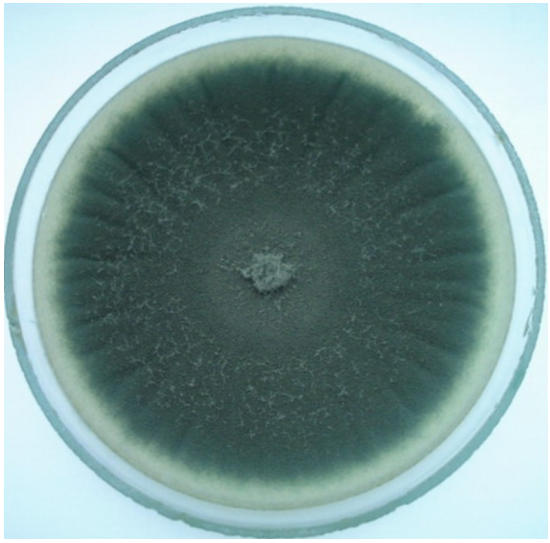
The *in vitro* activity of fluconazole, voriconazole and new triazole compound MXP-4509 on 273 yeast strains tested

Specie (isolates n)	Antifungal agent	MIC (mg/L)			
		Conc. range	MIC_{50}	MIC_{90}	Geometric mean
C. albicans (n=84)	FCA	0.125-0.5	0.25	0.5	0.205
	VOR	0.0156-0.0312	0.0156	0.0156	0.0159
	MXP	0.0156-0.0625	0.0156	0.0312	0.0194
C. parapsilosis (n=90)	FCA	0.125-2	0.5	1	0.535
	VOR	0.0156	0.0156	0.0156	0.0156
	MXP	0.0156-0.25	0.0625	0.0625	0.0484
C. glabrata (n=27)	FCA	0.25-64	32	64	17.281
	VOR	0.0156-8	0.125	8	0.340
	MXP	0.0156-8	0.125	4	0.231
C. pelliculosa (n=12)	FCA	2-8	4	ND	4
	VOR	0.0312-0.125	0.0625	ND	0.074
	MXP	0.0625-0.5	0.0625	ND	0.105
C. krusei (n=12)	FCA	16-64	32	ND	38.054
	VOR	0.25-0.5	0.25	ND	0.353
	MXP	0.25-1	0.5	ND	0.500
C. guilliermondii (n=9)	FCA	0.5-4	0.5	ND	1
	VOR	0.0156-0.0312	0.0156	ND	0.0196
	MXP	0.0625-0.25	0.25	ND	0.157
C. lusitaniae (n=6)	FCA	0.125-1	ND	ND	0.353
	VOR	0.0156	ND	ND	0.0156
	MXP	0.0156	ND	ND	0.0156
C. tropicalis (n=6)	FCA	0.5-64	ND	ND	5.656
	VOR	0.0156-2	ND	ND	0.176
	MXP	0.0625-8	ND	ND	0.707
S. cerevisiae (n=6)	FCA	4-16	ND	ND	8
	VOR	0.0156-0.0625	ND	ND	0.0312
	MXP	0.0312-0.0625	ND	ND	0.0441
T. asahii (n=6)	FCA	4-32	ND	ND	11.313
	VOR	0.0156-0.25	ND	ND	0.0624
	MXP	0.0312	ND	ND	0.0312
C. dubliniensis (n=3)	FCA	0.0625-0.25	ND	ND	0.125
	VOR	0.0156	ND	ND	0.0156
	MXP	0.0156	ND	ND	0.0156
C. intermedia (n=3)	FCA	0.125-0.5	ND	ND	0.25
	VOR	0.0156	ND	ND	0.0156
	MXP	0.0156-0.0625	ND	ND	0.0312
C. neoformans (n=3)	FCA	2-8	ND	ND	4
	VOR	0.0156	ND	ND	0.0156
	MXP	0.0625-0.25	ND	ND	0.125
G. capitatum (n=3)	FCA	4	ND	ND	4
	VOR	0.0156-0.0625	ND	ND	0.0312
	MXP	0.0156	ND	ND	0.0156
R. mucilaginosa (n=3)	FCA	16-64	ND	ND	32
	VOR	0.5-2	ND	ND	1
	MXP	8	ND	ND	8

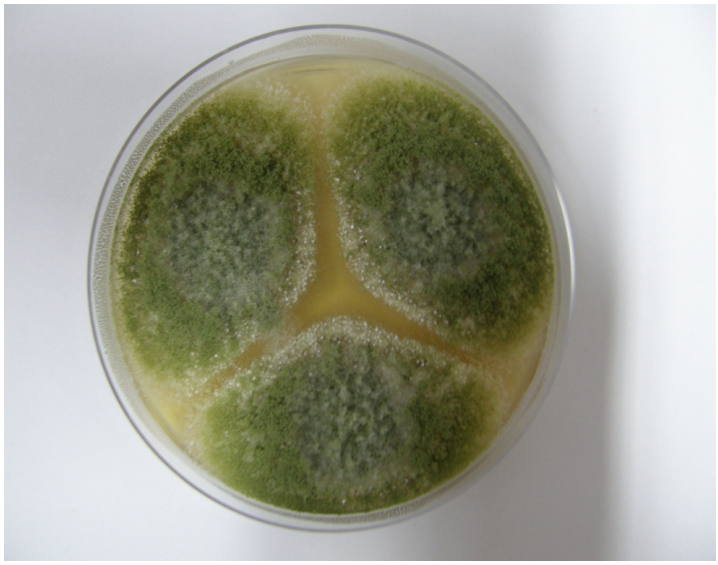
ASSESSMENT OF THE IN VITRO ANTIFUNGIC EFFECT OF MXP-4509 ON FILAMENTOUS FUNGI STRAINS USING EUCAST DEF.9.1 STANDARDISED METHOD

MATERIAL AND METHODS

Were studied 24 strains from *Aspergillus* genera. They are from laboratory collection, isolated from human patients with mycotic disorders: external ear disorder, skin disorder, traumatisms, invasive lung aspergillosis. From the total number of 24 strains, 2/3 were represented by *Aspergillus fumigatus* and 1/3 by *Aspergillus flavus*.



Aspergillus fumigatus colony aspect



Aspergillus flavus colony aspect

The only difference to previous described method is related to preparation of the inoculum and reading the results (vizually). Thus, the preparation of the inoculum is made from fresh and mature cultures (2-5 days), obtained on potato extract and glucose Agar. The colonies are mixed with approximately 5 ml sterile distilled water suplimented with 0.1% Tween 20. Then the conidias are carefully detached with a sterile swab and transferred with a pipette into a sterile tube. The suspension is homogenized with a vortex at 2000 rotation per minute for 15 seconds. The suspensions obtained are etalonated with a hemocytometer counting chamber. The inoculum must be examined to descover diverse hifae and conidial groups. If a semnificative number of hifae is detected ($\geq$ 5% from the fungic structures), 5 mL of suspension is transferred in a sterile seringe with a sterile filter attached having the pores dimension equal to 11µm. The newly obtained inoculum is collected and transferred into a sterile tube. This stage is necessary for *Aspergillus* species, eliminating the hifae and obtaining a homogenous suspension, formed only from spores. If agglomeration of hifae is observed after homogenization, it may be mixed again for 15 seconds. This technique may be repeated as many times it is necessary. The suspension is added with sterile distilled water to a 2-5x10^6 CFU/ml concentration. It follows a second dilution with sterile distilled water of 1:10 to a final inoculum.

The antifungal final concentrations were 16, 8, 4, 2, 1, 0.5, 0.25, 0.125, 0.625, 0.0312 mg/L. The growth degree from the cupules containing antifungal agent was read in comparison to the one from positive cupules (no antifungal agent added). Due to the fact that azole derivatives agents allow a residual growth of fungi into testing medium, an inhibition of 50% of growth is considered a satisfactory effect. The spectrophotometer reads the turbidity of each cupule and the obtained value will be compared with half of the value obtained for the growth positive sample, at a wave length of 405nm (that corresponds to a 50% growth inhibition). The minimal inhibitory concentration (MIC) was considered the lowest concentration that determines 50% growth inhibition (identical or lower turbidity than 50% of the one for positive sample).

Results

Table 4

MICs for 2 antifungal agents tested on *Aspergillus fumigatus* strains (n=16)

Concentration (mg/l)	**8**	**4**	**2**	**1**	**0.5**	**0.25**	**0.125**	**0.0625**	**0.0312**	**0.0156**
Voriconazole (strain n.)	4	5	1	6	0	0	0	0	0	0
MXP 4509 (strain n.)	2	1	0	2	1	0	0	0	0	0

Table 5

MICs for 2 antifungal agents tested on *Aspergillus flavus* strains (n=8)

Concentration (mg/l)	**8**	**4**	**2**	**1**	**0.5**	**0.25**	**0.125**	**0.0625**	**0.0312**	**0.0156**
Voriconazole (strain n.)	6	1	1	0	0	0	0	0	0	0
MXP 4509 (strain n.)	6	2	0	0	0	0	0	0	0	0

The two antifungal agents have a relatively similar effect on *Aspergillus* strains, emphysized by the close minimal inhibitory concentrations. All the tested strains have relatively high MICs of the tested antifungal agents that may indicate microbiologic resistance – an important prognostic factor for therapeutical susceptibility.

DETERMINATION OF THE LETHAL DOSE 90 IN MICE FOR THE CANDIDA ALBICANS SC5314 STRAIN

It was aimed to obtain a murine experimental model of disseminated candidemia (invasive) for ulterior tests regarding the therapeutical efficacy of the new MXP-4509 triazole. The main objective of the study was to determine the yeast dose which inoculated to outbred mice group, with approximately identical weigth, would produce death in 90% of the cases within 10-15 days period.

Material and method

- NMRi female mice, weightening 25 grames
- *Candida albicans* SC5314 (wild type) strain
- For preparation of the *Candida albicans* inoculum: YPD Agar dishes, inoculation loops, phosphate buffer, vortex mixer, sterile tubes, Neubauer improved counting chamber.
- For animal manouvers: scrub, mask, latex gloves, rubber ring for finger
- For intravenous inoculation of the mice: contention device for mice and 25 G serynge

Experimental design

The following parameters will be determined during infection period (up to 15 days): survival average time, the number of death mice versus the total number of infected mice.

DAY 0

The test strains were cultured on YPD Agar (yeast extract, peptone, glucose) and incubated at 30ºC for 24 hours.

DAY 1

Preparation of the inoculum (from *Candida albicans* strain):

- An yeast suspension is prepared in phosphate buffer and adjusted (by enumeration in counting chamber) to a turbidity equivalent to $1,5x10^6$, $2x10^6$, $2,5x10^6$, $3x10^6$ cells/ml (exact density is compulsory in this sort of experiment)

Inoculation of the mice:

- Are formed 4 groups with 25 animals each (a group corresponding to each turbidity of the inoculum)
- The mice are placed in contention devices
- The mice are heaten under infrared bulb lamp (in environment with low temperature the tail veins are colabated and the inoculation is difficult); the temperature is not exceeding 30º C for mice to avoid overheating
- An insulin serynge was filled with 550 microliters from the warm inoculum and the gas bubbles are removed from the 25 G needle adapted to the serynge
- The tail of the mice is fixed between the index and thumb finger from the left hand, then the needle is pointing the vein paralely with the blood vassel; it starts from the tip of the tail, if this fails it may restart the inoculation in an area near the bottom of the tail
- After inoculation, the needle is kept inside for approximately 5 seconds, thus the injected substance will be drained from the tail
- The inoculated mice are placed in the initial box where wil be fed and have the same microclimate conditions for 15 days, marking daily the deceased animals.

DAYS 2-10

- The inoculated mice are daily monitorised and are marked the deceased animals per days and groups.

Results

Analyzing the mortality dynamics for the 4 groups of inoculated mice was established that the dose of $2,5x10^6$ CFU/ml determined 90% mortality in 14 days (only 3 mice survived out of the total of 25 inoculated mice).