Project CNCSIS PN II – RU: TE_159/2010

EVALUATION OF THE ANTIFUNGAL EFFECT OF NANO CONJUGATES OF A NEW PROPICONAZOLE DERIVATIVE WITH BETA-CYCLODEXTRIN

Stage Report 2010

Objective: In vitro testing of the antifungal effect of the nanoconjugates

Activity: Taxonomical identification of the clinical strains from the personal collection of microorganisms

1. Materials and Methods

1.1. *Materials* were represented by 326 yeast and filamentous fungi strains, representing clinical isolates, which were identified by appreciation of the macro- and microscopically characters, physical and biochemical tests. All the strains were conserved at -80ºC for further tests.

1.2. *Equipments, reactive substances and devices*

- Exudates sterile swabs
- Culture media: Sabouraud broth (Merck, Germania), Potatoes Dextrose Agar (Merck, Germany), Sabouraud Chloramphenicol Agar (Biokar Diagnostics, France)
- Chloride acid, 1N solution
- Physiologic saline solution (0,85% NaCl)
- Blastesis Medium (Bio-rad, France)
- Gram colouring kit
- McFarland densitometer kit (bioMérieux, France)
- ID32C strips for yeast identification (bioMérieux, France)
- Micropipettes, automated pipette
- Densimat (bioMérieux, France)
- Thermal bath (Memmert, Germany)
- Optical binocular microscope (Micros, Austria)
- Incubator BE200 (Memmert, Germany)
- APIweb 1.2.1 soft for reading (bioMérieux, France)
- CandiSelect medium (Bio-rad, France)
- Anilin lactophenol blue (Merck, Germany)
- Microbiological calibrated loops

1.3. *Methods*

a) *purification of the isolates*. The isolated strains were cultured on specific media and incubated 24-48 hours at 36º ± 1ºC (for yeasts) or 72-96 hours at 30º ± 1ºC (filamentous fungi). The yeasts were Gram stained to observe bacterial contamination that would jeopardize the ulterior stage for specie identification. The ones identified to be bacterially contaminated, after microscopic examination of the Gram stained smear, were

inoculated in Sabouraud broth with additional chloride acid, 1N solution, as is presented in figure 1. The strains were incubated again for 24-48 hours at 36º ± 1ºC, and then cultivated on CandiSelect starting from the culture which grew on the tube with the highest quantity of chloride acid 1N solution. The CandiSelect media allows for mixed yeast culture to grow formed from one or more species. The purified tubes were used for identification tests for each species. For the filamentous fungi strains, from the obtained cultures, a single isolated strain was used that was transferred on a new medium and used for morphological tests identifications.

b) *yeast identification*. Was performed as it is described in figure 2.

- *germ tube test (blastesis test).* In tubes containing Blastesis medium, were added 0.5 ml yeast suspension and the tubes were incubated in Thermal Bath for 2 hours at 36ºC. The yeast suspension was prepared in saline physiologic solution, with a turbidity equivalent to 0.5 Mc Farland (approximately 10^6 cells/ml). After 2 hours incubation, the containing of the tubes were homogenised and a volume of 25 µl was inoculated on a slide mixed with a similar volume of lactophenol cotton blue. The extemporaneous sample was microscopically examined (x400) to determine the characteristic germ tubes for *Candida albicans* and *Candida dubliniensis* strains. As positive strains for germ tubes, each time was used *Candida albicans* ATCC 10231 type strain (figure 3).

- *incubation of the isolates at 45ºC* – as differential method for *Candida albicans* and *Candida dubliniensis* strains – is a test that needs more evaluations from specificity and sensibility point of view. Though, this test was used for a judicious differentiation of the isolates, due to its simplicity. The strains that tolerate the temperature of 45ºC for grow, belong to *Candida albicans* specie.

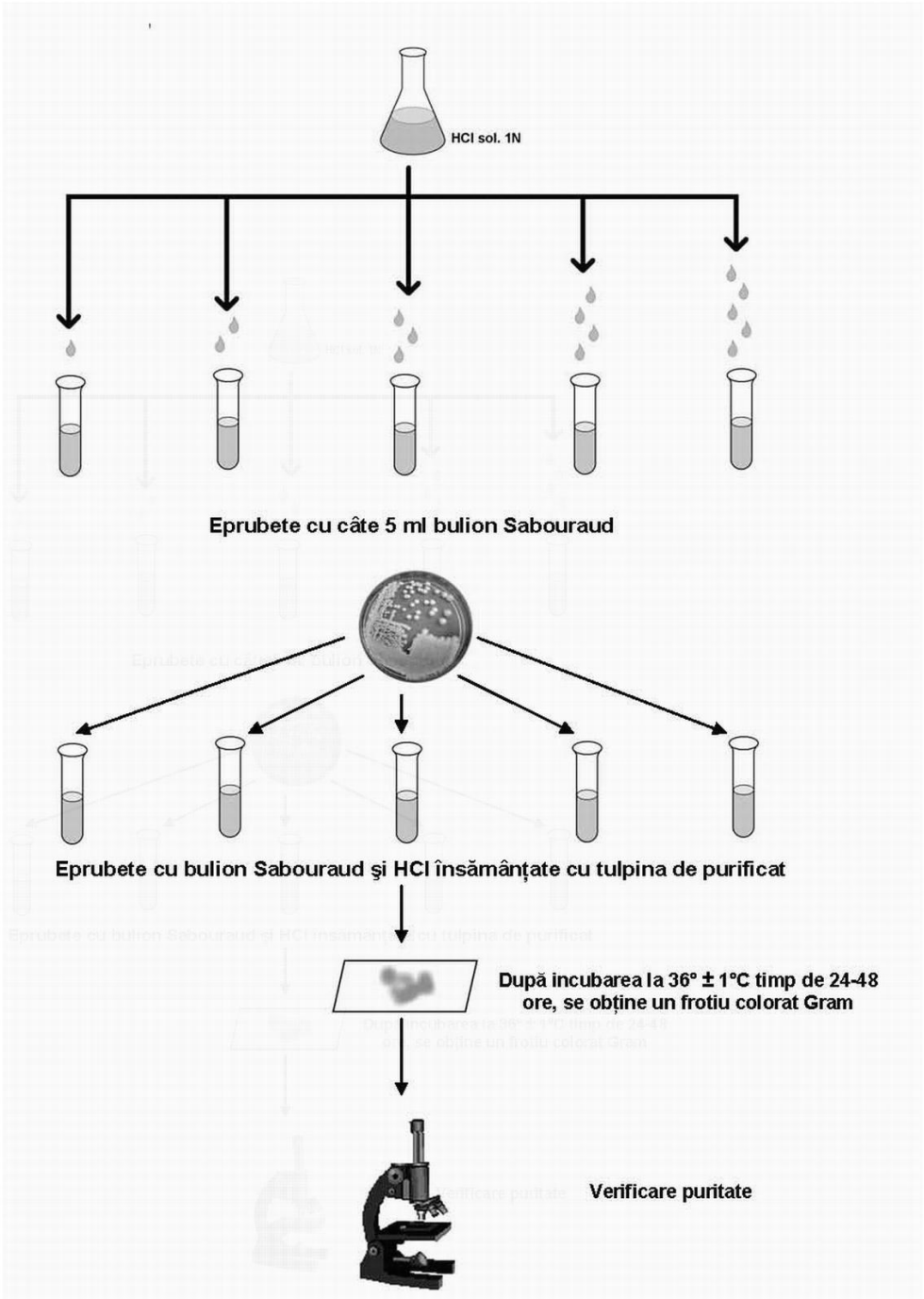

Figure 1. Purification of the isolates – method

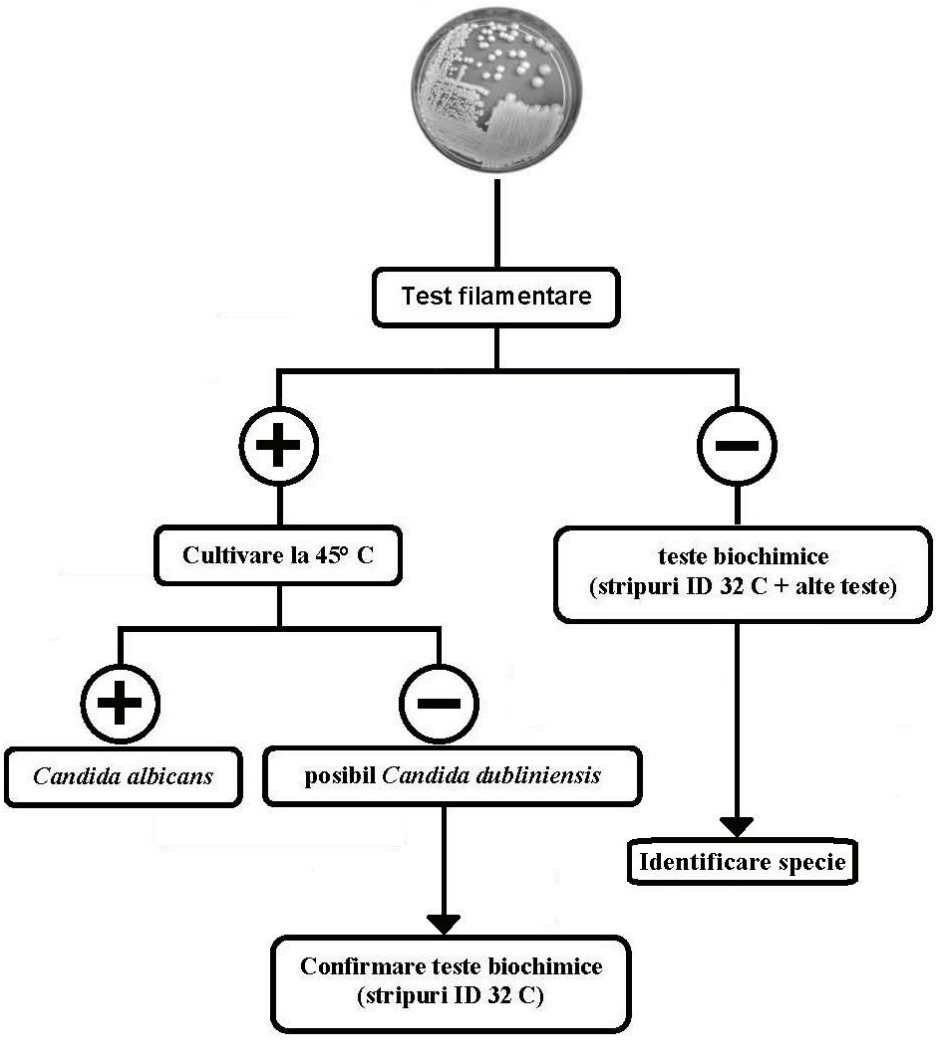

Figure 2. Algorithm for identification of the isolates.

- *biochemical tests.* The strains that did not produce germ tubes after incubation in Blastesis Medium were considered non-*albicans*/non-*dubliniensis* strains belonging to *Candida* genus or belonging to other yeast genera, fact that led to investigation of the biochemical phenotype to identify them. Thus, ID32C strips were used – a standardised system for yeast identification consisting of 32 tests for various carbon sources assimilation. Each cupule of the ID32C strips contains a unique carbon source, in a pre-established quantity.

Preparation of the inoculum is made in two stages:

- Preparation of the yeast suspension to a turbidity equivalent to 2 McFarland, using purified yeast cultures, 20-24 hours old, *API*® *Suspension Medium* tubes and *Densimat* device.

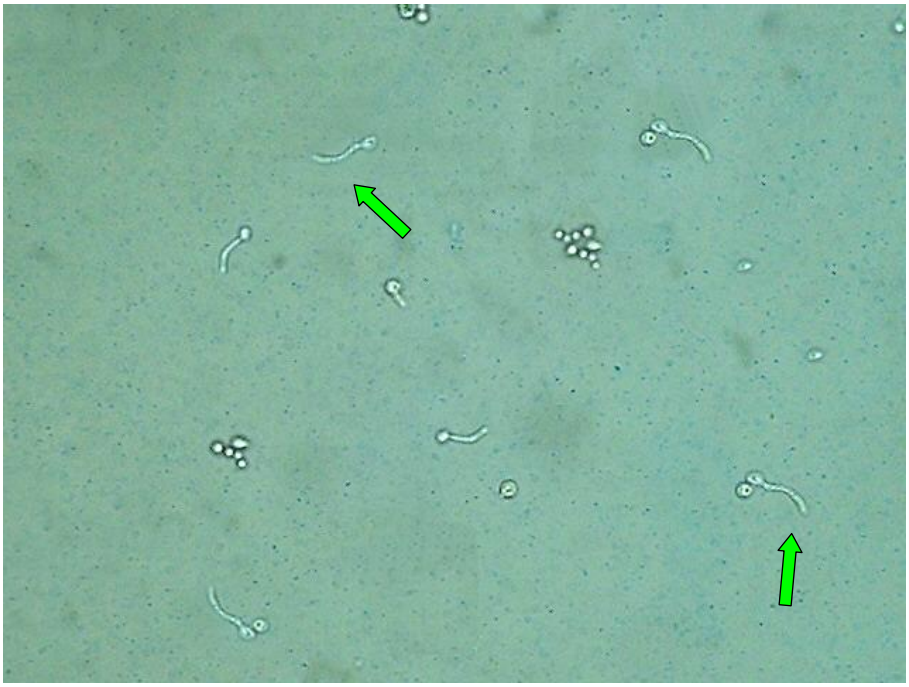

Figure 3. *Candida albicans* – germ tubes
(extemporaneous smear with Amann lactophenol cotton blue); x 400

- Inoculation of the minimal semisolid medium (*API® C Medium*) with a yeast suspension volume equal to 250 µl, homogenised using an automated *ATB®* pipette. The working technique is presented in figure 4.

Inoculation of the ID32C strips was made immediately after the preparation of the inoculum, by distributing a volume of 135 µl in each cupule of the strip, using the *ATB®* electronic pipette (figure 5). The inoculated strips were covered by a protection lid and incubated at 30°±1°C for 24-48 hours.

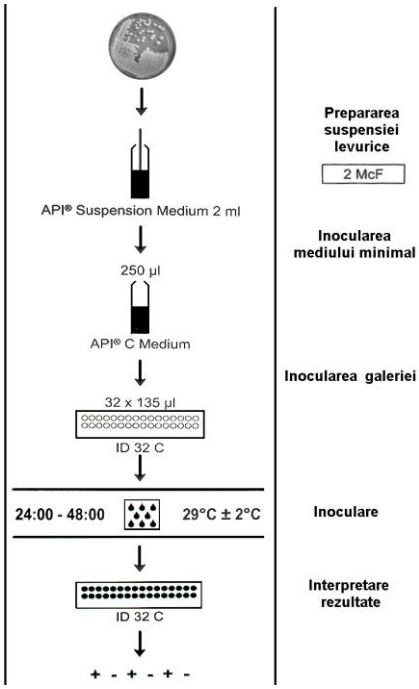

Figure 4. Inoculation of the ID 32C strips

Reading and interpretation of the results was automatically performed after 24 hours of incubation, using *APIweb 1.2.1 soft* for on-line identification of yeast strains (figure 6). The strips for which the indication of the *soft* were "*low discrimination*", "*doubtful or unacceptable profile*" or "*invalid identification before 48 hours*", were incubated for 24 hours more at 30°±1°C and the results interpretation remade after the time expired. In case when the *APIweb soft* indicated some supplementary tests, these were performed to assure a high accuracy of the identification.

c) *identification of the filamentous fungi*. Was made by corroboration of the macroscopically features (aspect of the colony, growth speed, colour of recto and verso of the colony) and microscopically features (the morphology of the fruiting bodies and spores, conidiogenesis type and their dimension). For microscopic examination there were used extemporaneous smears and scotch test, using as dissociative liquid – Amann lactophenol cotton blue.

Figure 5. Preparation and inoculation of the ID32C strips

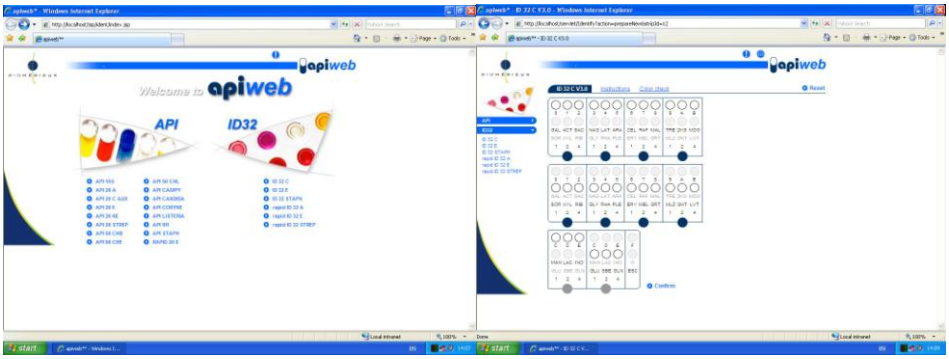

Figure 6. Display of the APIweb 1.2.1 soft

2. Results

After performing all the tests on the fungal strains, there were isolated the following species that will provide the material for future tests of the new propiconazole derivative with beta-cyclodextrin (MXP4509):

- *Acremonium sp.* (n=1)
- *Alternaria alternata* (n=1)
- *Aspergillus ochraceus* (n=1)
- *A. terreus* (n=1)
- *A. niger* (n=1)
- *A. flavus* (n=2)
- *A. fumigatus* (n=5)
- *Candida albicans* (n=99)
- *C. krusei* (n=14)
- *C. parapsilosis* (n=48)
- *C. pelliculosa* (n=4)
- *C. glabrata* (n=24)
- *C. dubliniensis* (n=2)
- *C. intermedia* (n=3)
- *C. guilliermondii* (n=9)
- *C. kefyr* (n=14)
- *C. inconspicua/norvegensis* (n=5)
- *C. lipolytica* (n=9)
- *C. lusitaniae* (n=3)
- *C. lambica* (n=5)
- *C. tropicalis* (n=5)
- *C. sphaerica* (n=8)
- *C. pulcherrima* (n=1)
- *C. valida* (n=3)
- *C. zeylanoides* (n=3)
- *C. famata* (n=2)
- *C. colliculosa* (n=3)
- *C. catenulata* (n=1)
- *C. sake* (n=2)
- *Cryptococcus curvatus* (n=2)
- *C. laurentii* (n=2)
- *C. neoformans* (n=4)
- *Epidermophyton floccosum* (n=1)
- *Geotrichum capitatum* (n=8)
- *Microascus cirrosus* (n=1)
- *Microsporum audouinii* (n=1)
- *M. gypseum* (n=1)
- *M. cookey* (n=1)
- *M. persicolor* (n=1)
- *Penicillium citrinum* (n=1)
- *Rhizopus stolonifer* (n=1)
- *Rhodotorula mucilaginosa* (n=2)
- *Saccharomyces cerevisiae* (n=7)
- *Trichosporon asahii* (n=8)
- *T. inkin* (n=1)
- *T. mucoides* (n=1)
- *Trichophyton rubrum* (n=1)
- *T. mentagrophytes* (n=1)
- *T. terrestre* (n=1)
- *T. ajelloi* (n=1)