

The Effect of Capsular Size of *C. neoformans* on Pathogenicity and Pathological Changes in Experimentally Infected Mice

¹Kawkab A. Ahmed, ²Heidy Abo El Yazeed, ³Hosam Kotb, ⁴Mona Elshabrawy, ^{4*}Randa Alarousy and ²Mohamed Refai

¹Department of Pathology, Faculty of Veterinary Medicine, Cairo University, Giza, Egypt

²Department of Microbiology, Faculty of Veterinary Medicine, Cairo University, Giza, Egypt

³Department of Reproductive Diseases, Animal Reproduction Research Institute

⁴Department of Microbiology and Immunology, National Research Centre, Giza, Egypt

*randaarousi@yahoo.com

Abstract: The present work was carried out to study the correlation of capsular size and pathogenicity of *C. neoformans* in experimentally infected mice. Various capsular sizes were induced by growing the standard strain of *C. neoformans* on media containing rosemary or thyme oil. Experimental infection of mice with the different phenotypes revealed that phenotype R with the large capsule caused signs of depression, loss of appetite and weight and the P. M. findings in this group of animals showed congested brain, haemorrhagic liver, congested lungs and splenomegally. when the mice were injected with cinnamon extract 24 hours post infection with the R phenotype. All mice showed normal activity, normal P. M. findings and clean histopathological profile for internal organs.

[Kawkab A. Ahmed, Heidy Abo El Yazeed, Hosam Kotb, Mona Elshabrawy, Randa Alarousy and Mohamed Refai

The Effect of Capsular Size of *C. neoformans* on Pathogenicity and Pathological Changes in Experimentally Infected Mice. Journal of American Science 2011;7(11):233-239].(ISSN: 1545-1003).

<http://www.americanscience.org>.

Key words: *C. neoformans*, Experimental infection, Pathological findings

1. Introduction

The polysaccharide capsule is a major virulence factor of *C. neoformans*. The effect of the *C. neoformans* polysaccharide capsule on the host cells can be summarized as follows: it interferes with phagocytosis, blocks the recruitment of inflammatory cells, increases co-stimulatory molecules, suppresses the delayed-type-hypersensitivity response, and reduces the antibody production in response to fungal infection (Vecchiarelli, 2003).

The capsule, as well as the soluble polysaccharide released from the yeast cells during infection, plays a significant role in pathogenicity, because it protects the yeast cells from phagocytosis and from cytokines induced by the phagocytic process and suppresses both cellular and humoral immunity (Drouhet and Segretain, 1951; McGaw and Kozel, 1979 and Kozel and Gotschlich, 1982). A large capsule can block the opsonic effect of complement and anticryptococcal antibodies. It can limit production of nitric oxide (which is an inhibitor of cryptococcal cells) and interferes with the antigen presentation process (Murphy, 1996).

Studies on the role of the capsule in the interaction between *C. neoformans* and macrophages resulted in compelling findings, which were described by Harrison and Levitz (2002). Once it is internalized by macrophages, *C. neoformans* may grow and eventually lyses the macrophage, be killed by the macrophage, or live within the macrophage in

equilibrium for an undetermined amount of time. In the lungs, all three outcomes are possible. Interestingly, the capsule plays an important role not only in the inhibition of phagocytosis but also in the inhibition of killing by macrophages.

Acapsular cryptococcal strains are not pathogenic (Chang and Kwon-Chung, 1994). On the other hand, although other cryptococcal species produce a similar capsule, they also are not pathogenic (De Baets *et al.*, 2002). This suggested that the capsule is necessary but not sufficient for fungal cells to cause disease and that its ability to aid infection is enhanced by other virulence factors. This hypothesis was supported by reports showing that during human cryptococcosis, acapsular or poorly encapsulated strains can be isolated. Other cases of isolation of *C. neoformans* acapsular or small-capsule strains from patients with cryptococcosis have been reported (Harding *et al.*, 1979; Ro *et al.*, 1987 and Salkowski and Balish, 1991).

The aim of the present work was today the correlation of capsular size and pathogenicity of *C. neoformans* in experimentally infected mice.

2. Materials and methods

Strains:

The standard strains of *C. neoformans* var. *grubii* serotypes A and var. *gattii* serotype C (Reg. no. 2041) were obtained from Kotb's collection.

Local strain (K 18) was kindly supplied by

veterinarian Shima Yousef Abou-Elmagd. The strain was a pink – pigmented colony isolated from chicken droppings and confirmed in Animal Reproduction Researches Institute (ARRI), Giza, Egypt. This strain was examined as (P- phenotype).

Oil agar media: (Alarousy, 2004)

Dextrose 40.0 g

Peptone 10.0 g

Agar 20.0 g

Chloramphenicol 50.0 g

Distilled water up to 1000.0 ml

Agar base was sterilized by autoclaving at 121 ° C, 15 lb / in² for 20 min.

Rosemary oil or thyme oil was sterilized by filtration before it had been added to the medium in a final concentration of 10%.

Experimental animals (mice):

Seventy male mice weighing 80 g each were housed and supplied with a marketed ration and water. They were kept under observation for 1 week before starting the experiment

Material for histopathological examination:

- Formalin 10% (adwic) : absolute was diluted to 10% concentration by means of distilled water
- Ethly alcohol 70% -80% -90% -100% (Adwic) : absolute ethanol was diluted to 70% 80% and 90% concentration by means of distilled water
- Xylene solution (Adwic).
- Paraffin
- Hematoxylen and eosin (H&E) stain
- Gomori Silver stain (GMS).
- Egg albumin

Preparation of *C. neoformans* phenotypes

- The original phenotype (O) was obtained by growing the reference strain (2041) on Sabouraud Dextrose agar medium with chloramphenicol.
- The large sized capsular phenotype (R) was obtained by growing the reference strain (2041) on rosemary oil / SDA medium with chloramphenicol.
- The small sized capsular phenotype (T) was obtained by growing the reference strain (2041) on thyme oil / SDA medium with chloramphenicol.
- Acapsular hyphal form phenotype (H) was obtained by prolonged preservation of the original reference strain (2041) (serotype A) in lyophilized form.
- The pink-coloured phenotype (P) was obtained by growing the isolated strain on SDA medium with chloramphenicol.

Experimental infection:

Seven groups of male mice, weighing about 80 gram each were used; each group of them contained 10 mice. Six groups were used for the proper experimental infection and the seventh group was kept as a control. Yeast concentrations were adjusted using the haemocytometer to count the yeast cells (1×10^5 - 1×10^7 / ml) in order to equalize the injected yeast cells rather than the capsule size.

Each mice of the first 4 groups was injected with 200 µl yeast cells suspension (I / V) of the respective phenotype (O, R , T and H). The fifth group (R') was injected with 200 µl of cinnamon bark extract 24 hour post infection with the (R) phenotype and the sixth was injected with 200 µl of (P) suspension (pink colony phenotype). The last group was injected with sterile normal saline solution (I/V) and kept as a control.

Clinical and P. M. examinations

All the mice were observed to detect any abnormal clinical or behavioral signs as depression, off food or loss of appetite, over activity, nervous manifestations, weight loss or emaciation or swelling or edema in head.

Mice were observed daily for any sign of disease or abnormalities.

Activity, weight loss or any clinical abnormalities appeared on mice were recorded. There were no deaths among mice. All mice from all groups were euthanized by the elapse of the second week post infection and their organs; brain, liver, spleen and lung were extracted for histopathological examination, and post mortem abnormalities of the extracted organs were recorded.

Histopathological studies

Specimens from brain, liver, spleen and lung were taken immediately after sacrifice from all groups. Specimens were fixed in 10% buffered neutral formalin solution. The fixed specimens were then trimmed, washed and dehydrated in ascending grade of alcohol cleaned in Xylene embedded in paraffin sectioned (4-6 microliter thickness) and stained with Gomori silver stain (GMS), hematoxylen and eosin as routine stain

3. Results

P. M. findings of experimentally infected mice:

All mice remained alive during the 2 weeks of observation. The animals also infected with phenotype O strain showed normal activity, while those infected with phenotype R showed signs of depression, loss of appetite and weight.

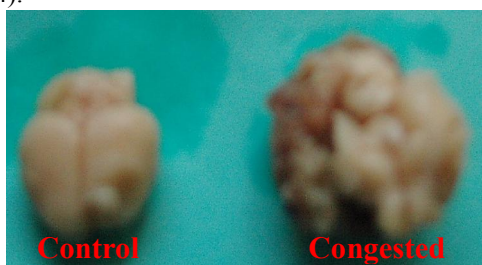
The P. M examination revealed pale liver containing pus sac in mice infected with phenotype O (Photo 1), only slight deviations from the normal were seen in brain and lung as well as moderate enlargement of the spleen.



Control Pale liver

Photo (1): Pale liver containing pus sac in mice infected with original strain (O)

Mice of group R showed congested brain, haemorrhagic liver, congested lungs and moderate splenomegaly (Photos 2 & 3 & 4).



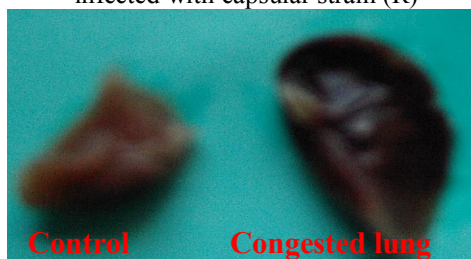
Control Congested

Photo 2 Congested brain in mice infected with capsular strain (R)



Control Congested liver

Photo 3 Congested liver in mice infected with capsular strain (R)



Control Congested lung

Photo 4 Severe congested lung in mice infected with capsular strain (R)



Control Severe splenomegally

Photo 5 Severe spleen enlargement in mice infected with a-capsular strain (T)

All mice of R' group injected with cinnamon extract 24 hours post infection with yeast cells showed normal activity, normal P. M. findings and clear histopathological profile for internal organs.

The mice of group T showed signs of depression, loss of appetite and weight. The brain was congested; liver showed slight enlargement and dull appearance; lungs were slightly oedematous with mucoid material and spleen showed severe enlargement of the spleen (Photo 5) and liver. Mice of groups P and H showed normal activity. P. M. findings were mainly moderate to severe splenomegally.

Microscopical appearance of *Cryptococcus neoformans* in the tissue:

In H&E stained tissue sections, the yeast cells of *Cryptococcus neoformans* stain pale blue to pink and ranged from 2 – 20 μm in diameter. They have relatively thin walls and are very pleomorphic, appearing round, oval, elliptical and crescent or cup – shaped cells of many shapes and sizes often are present in a single microscopic field.

The capsule of poorly encapsulated cells (as in phenotype T) may not be seen. The mucopolysaccharide capsules where obvious, appears as smoothly contoured, clear, unstained space or —halo surrounding the yeast cells. It does not stain with H&E.

The stained capsules may have a spinous or scalloped appearance as the result of the irregular contraction of the capsular material during formalin fixation. Cryptococci are also well demonstrated with the special fungus stains that colour the entire fungal cells. The number and distribution of fungal cells and the tissue response that they elicit vary from case to case.

Liver:

Microscopically, liver of control mice revealed the normal histological structure of hepatic lobule. Meanwhile, liver of mice from group (T) showed vacuolar degeneration of hepatocytes and

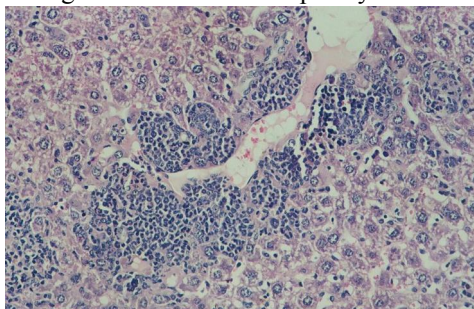


Photo (6): Liver of mice from group (T) cells infiltration (H and E stain X 200)

perivascular leucocytic cells infiltration mainly macrophages and epithelial cells (Photo 6). Moreover, liver of mice from group (O) showed vacuolar degeneration of hepatocytes, focal area of hepatic necrosis associated with leucytic cells infiltration and focal hepatic hemorrhage dispersed the hepatocytes from each other. Liver of mice from group (R) showed focal area of hepatic necrosis associated with leucocytic cells infiltration. However, liver of mice from group (R') showed only vacuolization of hepatocytes. By GMS stain, black stained wide capsule of yeast like *Cryptococcus* in the hepatic tissue of mice from group (R) could be demonstrated (Photo 7).

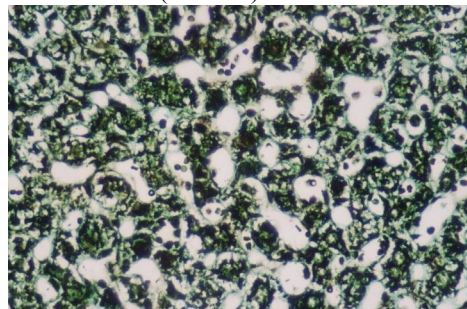


Photo (7): Liver of mice from group (R) showing perivascular leucocytic showing black stained wide capsule of yeast cells of *Cryptococcus* (GMS stain X 400)

Lung:

Microscopically, lung of control mice revealed no histopathological changes. Meanwhile, lung of mice from group (T) showed vacuolation of epithelial lining bronchiole, thickening of interstitial tissue with mononuclear inflammatory cells, desquamation of epithelial lining bronchiole with intraluminal soap – bubble yeast like *Cryptococcus* as well as focal infiltration with mononuclear inflammatory cells. Moreover, lung of mice from group (O) revealed soap – bubble appearance of *Cryptococcus* in the lumen of the bronchiole (Photo

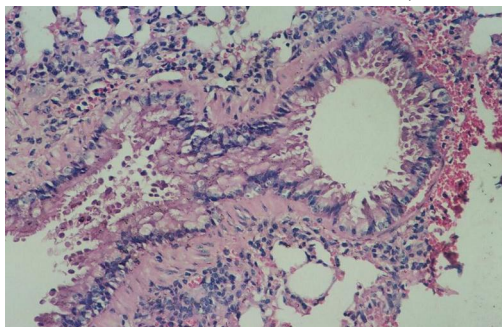


Photo (8): Lung of mice from group (O) showing soap – bubble appearance of *C. neoformans* in the lumen of the bronchiole (H and E stain X 200)

8), pulmonary hemorrhage and hemosiderosis. Examined lung of mice from group (R) revealed hyperplasia of epithelial lining bronchiole associated with desquamation of epithelium and intraluminal soap – bubble appearance due to masses of *Cryptococcus*. Meanwhile, lung of mice from group (R') showed apparent normal lung tissue. By GMS stain, can demonstrate *Cryptococcus* yeasts of all phenotypes which vary in size and shape (rounded and crescentic) with black stained wide capsule in the alveoli (Photo 9).

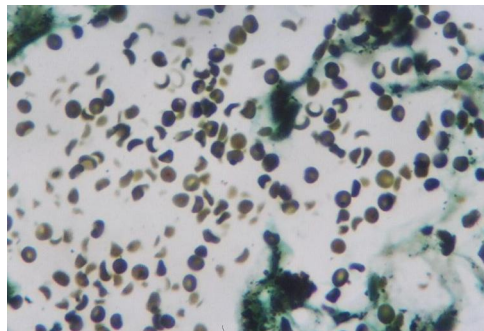


Photo (9): Lung of mice from group (R) showing round and crescent yeast cell of *Cryptococcus neoformans* with wide black capsule (GMS stain X 1000)

Spleen:

Microscopically. Spleen of control mice

revealed no histological changes. Meanwhile, spleen of mice from group (T) showed deposition of eosinophilic protein material in the red pulp of the spleen. However, some examined spleen of mice from group (O) revealed no histological changes, while presence of multiple of megakaryocytes was noticed in examined spleen sections from the same previous group (O). No histological changes were noticed from spleen of mice from groups (R & R').

Brain:

Microscopically, brain of control mice revealed no histological changes. Meanwhile, brain of mice from group (T) showed neuronal degeneration, neuronophagia and focal gliosis (focal proliferation of glia cells). On the other hand, brain of mice from group (O) revealed pyknosis of neurons, perivascular cuffing with mononuclear cells, as well as marked cerebral hemorrhage. However, examined sections from group (R) showed pyknosis of neurons, cerebral hemorrhage and hemorrhage in Virchow Rubin space. Meanwhile, brain of mice from group (R') showed improvement in the histological picture as the brain sections showed only pyknosis of some neurons. By GMS stain we can demonstrate the yeast like *Cryptococcus* with black stained wide capsule (Photo 10).

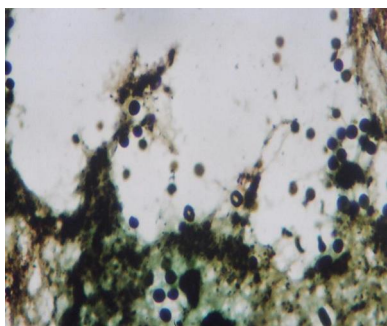


Photo (10): Brain of mice from group (O) showing black stained wide capsule of yeast cells of *Cryptococcus neoformans* (GMS stain X1000)

4. Discussion

Although the tissue reaction in cryptococcosis varied considerably from organ to organ and from case to case, generally the difference was minimal. Frequently, only tissue macrophages were found, but in rare cases there maybe many giant cells and dense infiltration of plasma cells and lymphocytes. The livers of mice infected with phenotype (T) showed **granulomatous** tissue reaction which was composed of histocytes, giant cells, lymphocytes, some fibroblastic activity and yeast cells which were few and found within giant cells and histocytes. Another

tissue reaction pattern, **gelatinous** one was also observed in the lungs of mice infected with original and large capsule size phenotypes (O) and (R) phenotypes respectively, gelatinous pattern was composed of masses of yeast cells, mucoid degeneration of the invaded tissue giving the appearance of soap – like appearance, while yeast cells in this pattern were found freely in the tissue (**Small et al., 1986**). During experimental infection done in the current study, there weren't any mortality among the infected mice and this was explained by **Small et al. (1986)** as the capsule is necessary but not sufficient for fungal cells to cause disease, as its ability to aid infection is enhanced by other factors. Because *C. neoformans* varies greatly in size and shape, and its encapsulated forms are not always prominent, cryptococcosis should be considered in the histologic differential diagnosis of virtually any yeast infection. Generally, poorly encapsulated intracellular cryptococci stimulate a granulomatous inflammatory reaction (**Farmer and Komorowski, 1973**). Cryptococcal granulomas are composed of varying numbers of histocytes, giant cells, lymphocytes, plasma cells, neutrophils and fibroblasts. The tissue response to cryptococci is typically non suppurative. So, Neural generation was observed in brains of infected mice and their livers. Focal proliferation of glia cells (focal gliosis) was seen in brains, livers and lungs of mice infected with phenotype (T).

These features were not only observed in organs of mice infected with phenotype (T), but they were also seen in organs of those infected with phenotypes (P) and (H).

No haemorrhage was observed in organs of mice infected with phenotypes (T), (P) and (H), while in phenotypes (O) and (R) it was seen clearly. Necrosis was seen in most of infected cases and piknosis of neurons due to cell death was seen in phenotypes (O) and (R). Beside piknosis, vacuolar degeneration, hyperplasia associated with desquamation of epithelial lining of bronchioles were observed in these phenotypes – infected groups (O&R) and intraluminal soap – bubble appearance due to profuse masses of *Cryptococcus neoformans* was detected in all the examined tissues beside, perivascular leucocytic cell infiltration was seen in all examined organs.

According to **Blackstock and Murphy (1997) and Blackstock et al. (1999)**, although the strain with reduced virulence was not able to increase its capsule size in the presence of CO₂ as a factor aid in increasing the capsule size. That strain elicited a rapid inflammatory response in the host and was efficiently cleared from the lungs. Since many reports provided strong evidence that capsule

modulation is a process that plays an important role during infection. Several studies had found, however, that inducing infection with modulated strain with a large capsule gives no correlations with virulence (**Littman and Tsubura, 1959; Dykstra et al., 1977 and Clancy et al., 2006**). This lack of correlation is most probably due to the fact that capsule size does not predict the degree of encapsulation of a strain during infection. Furthermore, *C. neoformans* has multiple virulence factors that contribute to the overall virulent phenotype and it is possible that differences in non – capsular virulence factors expression affects the overall virulence potential to obscure a correlation with capsule size (**MacClelland et al., 2006**).

In addition, capsule size during infection varies according to the infected organ, the lung being the place where higher capsule size is found, which indicates that micro environments during infection have a great impact on the capsule size (**Rivera et al., 1998**).

Finally, the results obtained in the present study confirmed the fungicidal effect of cinnamon bark extract and thus we may nominate cinnamon as commercial fungicidal agent and this is substantiated by the work of **Hammer - Schmidt et al. (1993) and Hili et al., 1997**.

Corresponding author

Randa Alarousy
Department of Microbiology and Immunology,
National Research Centre, Giza, Egypt
randaarousi@yahoo.com

References

Vecchiarelli, A.; Pietrella, D.; Lupo, P. ; Bistoni F.; McFadden, D. C. and Casadevall. A. (2003). The polysaccharide capsule of *Cryptococcus neoformans* interferes with human dendritic cell maturation and activation. *J. Leukoc. Biol.* 74:370-378.

Drouhet, E. and Segretain, G. (1951). Inhibition de la migration leucocytaire *in vitro* par Un polyoside capsulaire de *Torulopsis (Cryptococcus) neoformans*. *Ann. Inst. Pasteur (Paris)*; 81(6):674–676.

McGaw, T. G. and Kozel, T. R. (1979): Opsonization of *Cryptococcus neoformans* by human immunoglobulin G by cryptococcal polysaccharide. *Infect. Immun.* 25:262–267.

Kozel, T. R. and Gotschlich, E. C. (1982): The capsule of *C. neoformans* passively inhibits phagocytosis of the yeast by macrophages. *J. Immunol.*, 129: 1675-1680.

Murphy, J. W. (1996). Slick ways *Cryptococcus neoformans* foils host defenses. *ASM News* 62: 77.

Harrison, T. S., and Levitz, S. M. (2002). *Cryptococcus neoformans* and macrophages. Marcel Dekker, Inc., New York, N.Y.

Chang Y. C., Kwon-Chung K. J. (1994): Complementation of a capsule-deficient mutation of *Cryptococcus neoformans* restores its virulence. *Mol. Cell Biol.* 14:4912–4919.

Harding, S. A.; Scheld, W. M. ; Feldman, P. S. and Sande, M. A. (1979). Pulmonary infection with capsule-deficient *Cryptococcus neoformans*. *Virchows Arch. A. Pathol. Anat. Histol.* 382:113-118.

Ro, J. Y.; Lee, S. S. and Ayala, A. G. (1987) Advantage of Fontana-Masson stain in capsule-deficient cryptococcal infection. *Arch. Pathol. Lab. Med.* 111:53-57.

Salkowski, C. A., and Balish, E. (1991). Susceptibility of congenitally immunodeficient mice to a nonencapsulated strain of *Cryptococcus neoformans*. *Can. J. Microbiol.* 37:834-839.

Alarousy, R. M. (2004): Molecular biology studies on *Cryptococcus neoformans*. M. V. Sc. Thesis. Faculty of Veterinary Medicine, Cairo University, Giza, Egypt.

Small, J. M.; Mitchell, T. G. & Wheat, R. W. (1986). Strain variation in composition and molecular size of the capsular polysaccharide of *Cryptococcus neoformans* serotype A. *Infect. Immun.* 54, 735-741.

Farmer, S. G. and Komorowski, R. A. (1973). Histologic response to capsule – deficient *Cryptococcus neoformans*. *Arch. Pathol.* 96: 383 – 387.

Blackstock, R. and Murphy, J. W. (1997). "Secretion of the C3 component of complement by peritoneal cells cultured with encapsulated *Cryptococcus neoformans* " *Inf. Immunity* 65(10): 4114 – 4121.

Blackstock, R. B.; Buchanan, K. L.; Adesino, A. M. and Murphy, J. W. (1999). "Differential regulation of immune responses by highly and weakly virulent *C. neoformans* isolates." *Inf. immunity* 67(7): 3601 – 3609.

Littman, M. L. and Tsubura, E. (1959). "Effect of degree of encapsulation upon virulence of *Cryptococcus neoformans* " *Proc. Soc. Exp. Biol. Med.* 101: 773 - 777.

Dykstra, M. A.; Friedman, L. and Murphy, J. W. (1977). Capsule size of *Cryptococcus neoformans*: control and relationship to virulence. *Infect. Immun.* 16:129–135.

Clancy, C. J. N.; Alandoerffer, R.; Cheng, S.; Iczkowski, K.; Richardson, M. and Graybill, J. R. (2006). "*Cryptococcus neoformans* var. *grubii* isolates recovered from persons with AIDS demonstrates a wide range of virulence during murine meningoencephalitis that correlates with the

- expression of certain virulence factors." J. Microbiology 152(8): 2247 - 2255.
- MacClelland, E. E.; Bernhardt, P. and Casadevall, A. (2006). Estimating the relative contributions of virulence factors for pathogenic microbes. Infect. Immun. 74:1500-1504.
- Rivera, J.; Feldmesser, M.; Cammer, M. and Casadevall, A. (1998). Organ-dependent variation of capsule thickness in *Cryptococcus neoformans* during experimental murine infection. Infect. Immun. 66:5027-5030.
- Hammer-Schmidt, P.J.; Clark, A. M. and Soliman, F. M. (1993): Chemical composition and antimicrobial activity of essential oils of *Jasonia candicans* and *Jasonia montana*. Planta. Medica. 59: 68-78.
- Hili, P.; Evans, C. S. and Veness, R. G. (1997): Antimicrobial action of essential oils: the effect of dimethylsulphoxide on the activity of cinnamon oil. Lett. Appl. Microbiol. 24:269-275.

11/25/2011