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SHORT COMMUNICATION

Negative cross-reactivity of rabbit anti-*Malassezia furfur* antibodies with other yeasts

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Abstract

Anti-*Malassezia furfur* monospecific polyclonal antibodies was produced by repeated immunization of rabbit with *Malassezia furfur* yeast cells mixed with Freud adjuvant. The antibody titres of respective rabbit's serum samples prior to and after each immunization against *M. furfur* were assayed by indirect immunofluorescence technique using the *M. furfur* whole yeast antigen fixed in Teflon coated slides. The highest anti-M. furfur antibody titre achieved was 1 in 1280 dilution. At 1:20 dilution, none of the respective serum samples taken at various stages of immunization gave positive immunofluorescent staining against any of the other species of yeasts tested in this study. Anti-M. furfur monospecific polyclonal antibodies produced in rabbit in this study has the potential for diagnostic application in immunohistochemical detection of *M. furfur* in human tissues.

Key words: Malassezia furfur, antibodies, immunofluorescence.

Lipophilic yeasts, classified under the genus *Malassezia*, presently comprise 7 species.[1] As a group, its name has undergone many changes since its first description as the cause of pityriasis versicolor in 1846.[1-4] Its great morphological polymorphism and the lack of suitable simple culture media in the early period made the study of these lipophilic yeasts unsatisfactory.[5-7] With the development of International Medical University-Malassezia furfur (IMU-Mf) medium, these lipophilic yeasts could be easily cultured from the skin of both normal human as well as patients with pityriasis versicolor.[8]

An isolate of lipophilic yeast (226/02), isolated from a patient with pityriasis versicolor was subcultured in IMU-Mf medium for 3 days. A single yeast colony was selected from the subculture plate and confirmed to be *Malassezia furfur* by the molecular technique described in Makimura *et al*.[9] A loopful of yeasts from the same colony was transferred into 50 ml of liquid IMU-Mf medium in a sterile 250-ml conical culture flask and incubated at 35^{0}C in a shaking incubator. After 3 days of cultivation, the yeasts were pelleted by centrifugation at 1000 g for 10 minutes. The pellet was washed twice with 5% Triton-X by the process of resuspension and centrifugation. After the last Triton-X wash, the pellet was washed thrice with sterile phosphate buffered saline (PBS) by the same process of resuspension and centrifugation.

After the last PBS wash, the yeast pellet was mixed and suspended in 2 ml of Freud complete adjuvant and injected into the nape of an adult rabbit at 4 different sites with a volume of 0.5 ml at each site. A second immunization of the rabbit was carried out a month later with a similar quantity of *M. furfur* yeasts but in 2 ml of Freud incomplete adjuvant. Third immunization of the rabbit was carried out at 2 weeks after the second, with the same quantity of *M. furfur* yeast cells as the previous two but without any added adjuvant. Two milliliter of blood was collected by mini-venesection of the rabbit ear-lobe marginal vein prior to each immunization. The serum sample collected in each occasion was stored at -20^{0}C and the titre of anti-Malassezia antibodies was assayed at the end of the immunization.

A week after the 3rd immunization, the rabbit was noted to develop progressive hind limb paresis. The animal was subsequently euthanized a week later and all the required blood was

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collected by cardiac aspiration. The antibody titre of respective rabbit's serum samples taken at various stages of immunization against *M. furfur* were assayed by indirect immunofluorescence technique using the *M. furfur* whole yeast antigen fixed in Teflon coated slides which were prepared for an earlier seroprevalence study.[8] Briefly, the scale-up growth of the selected single colony of *M. furfur* yeast was carried out by spreading a suspension of the yeast in sterile phosphate buffered saline (PBS) on the surface of the IMU-Mf agar. After 48 hours of cultivation at 35^0C, the yeast cells were harvested and suspended in 10 ml of PBS containing 5% Triton-X for 10 minutes, with intermittent agitation to disperse the aggregated yeast cells. The yeast cells were then washed four times to remove the Triton-X using sterile PBS by centrifugation at 1000 g for 10 minutes. After the last wash, the yeast cells were suspended in sterile PBS at a concentration of 5000 cells per millilitre. Ten microlitre of the resultant yeast suspension was careful layered onto each well of the Teflon coated slides. The slides were air-dried over a warm plate and subsequently fixed in cold acetone for 10 minutes to be used as antigen for the detection and assay of anti-M. furfur antibodies. Five microlitre of each respective thawed serum was serially diluted at 2-fold dilution with sterile PBS starting from the initial dilution of 1:10 to 1:2560. 20 µl of each diluted serum sample was transferred onto each respective well of the Teflon coated slide. The slides with the applied test sera were incubated in a moist chamber for 30 minutes at 37^0C. Subsequently, the slides were rinsed with PBS before being soaked for a further 10 minutes in PBS solution that was kept in gentle motion by a magnetic stirrer. The slides were allowed to air dry over a warm plate and probed with 20 µl of 1:40 diluted fluorescein conjugated swine anti-rabbit IgG (Dako, USA). The slides were then incubated for another 30 minutes at 37^0C in a moist chamber. The same process of washing and drying was again carried after the second incubation. Following this, the slides were mounted with a commercially supplied mounting fluid and the titres of antibodies were read under a UV fluorescence microscope (Olympus BX50, Japan) at 400X magnification. The rabbit antibody titres against *M. furfur* prior to first, second and third immunization were at 0, 1:160, and 1:640 dilution respectively with an antibody titre of 1:1280 dilution at the time the rabbit was euthanized.

A number of known mycological yeasts were obtained from the culture collection of the Mycology Unit, Department of Medical Microbiology, University of Malaya. They were *Candida albican*, *C. tropicalis*, *C. dubliniensis*, *C. glabrata*, *C. parasilopsis*, *C. krusei*, *Rhodotorula rubrae*, *Cryptococcus neoforman*, *Geotricum*, *Trichosporon cutaneum*, and the yeast-form of *Penicillium marneiffei*. The species of yeasts were separately cultured in Sabouraud dextrose agar plates except for the *Penicillium marneiffei* which was cultured in blood agar. After 4 days of culture, they were harvested and antigen slides were prepared by the same process of washing with 5% Triton-X, followed by sterile PBS and finally fixed in Teflon coated slides as described above. All the rabbit's sera taken at different occasions were found to react weakly with Candida species at 1:10 dilution even the one prior to immunization. This was probably due to cross-reactive antibodies at low serum dilution or the rabbit used in this study was colonized by Candida species. However, none of the serum samples was found to give positive reaction by immunofluorescent staining at 1:20 dilution to all the species of other yeasts, including Candida species, tested in this study. Thus, anti-M. furfur monospecific polyclonal antibodies produced in rabbit in this study has the potential for diagnostic application in immunohistochemical staining for the detection of *M. furfur* antigen present in human tissues at a dilution of 1:40 or higher.

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