Prophylaxis and therapeutic ability of inactivated dermatophytic vaccine against dermatophytoses in rabbit as an animal model

Running title: Prophylaxis and therapeutic vaccine against dermatophytes

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31.01.2020
05.07.2020

Abstract
Objective: Dermatophytosis is a group of widely distributed cutaneous diseases in human and animals. It causes a serious infection in some cases in humans with an economic loss in farm animals. Investigation of prophylaxis and therapeutic vaccine against dermatophytosis is the main aim of this study.
Materials and Methods: Rabbit was chosen as an animal model for dermatophytosis in two parts of a case control study. Inactivated cells of *Trichophyton mentagrophytes* were prepared for use as a vaccine. The prophylaxis part included vaccination of rabbit with either of prepared vaccine alone or with Freund’s adjuvant, followed by infection with the same fungus. The second part included treatment of infected rabbit with an inactivated vaccine.
Results: Prepared vaccine showed prophylaxis ability against infection with *T. mentagrophytes* for more than 6 months without needing an adjuvant and also revealed the therapeutic ability in infected animals after a short time (16 days) compared with the control group.
Conclusion: Inactivated vaccine gives the animals durable protection and short-time treatment of infection with dermatophytosis.
Key words: Dermatophytosis; Trichophyton mentagrophytes; vaccine; prophylaxis; rabbit
Introduction
Fungal infections are widely distributed nowadays with serious mortality and morbidity rates all over the world [1]. Most of these infections, especially systemic types, are usually diagnosed too late for starting treatment [2]. Thus, prophylaxis by vaccination against most common fungal infections should take priority to limit the incidence of such diseases. For decades, antifungal vaccines have been considered impractical by most international scientific societies [1, 3-4]. Most attention is focusing on the development of vaccines against viral and bacterial infections [1,3]. The reason is that fungal infections usually show low incidence rates and some of them are not widely distributed in comparison with bacterial and viral infections [1]. Weakness in the immune system in most patients with fungal infections was also believed to decrease the efficacy of vaccines against fungi [3]. However, there has been no vaccine with a license for use against fungal infection in humans until now [4-5]. Recently, this view has changed due to growth of interest to limit a common type of fungal infection after its increasing incidence, especially in immunocompromised patients or those with other predisposing factors [1,6]. Several studies approved the suitability of vaccine development against common fungal infections such as those caused by Aspergillus spp., Candida spp., Paracoccidioides brasiliensis, Sporothrix spp., Cryptococcus spp., Coccioides spp., Histoplasma spp. and Blastomyces spp. [5-6].

Dermatophytosis is one of a group of common skin diseases in both human and animals [7-8]. It is caused by a special group of keratinophilic fungi called dermatophytes [7]. Although dermatophytosis is restricted within a cutaneous layer of the skin, a systemic distribution in human has been registered by many case studies [9-11]. This development in pathogenesis of dermatophytes directed specialists to consider dermatophytosis as a serious disease that needs more attention. In animals, dermatophytosis is considered a very important disease due to its effect on the economic value on animal breeding [12]. Although discovery of an effective vaccine against dermatophytosis is not a new idea, researchers are continuously trying to find a perfect one with good prophylaxis and therapeutic ability. Various components of dermatophytes have been evaluated as a vaccine against dermatophytosis, but without approval for commercial use in human [13-14]. However, these studies are still at an experimental level, even though a few of them are commercially used for various animals such as dogs, cats, bovines, and guinea pigs [13-14, 15-26]. Rabbits were chosen as a model to investigate the suitability of a vaccine from Trichophyton mentagrophytes for prophylaxis and treatment of dermatophytosis.

Materials and Methods
Fungal isolate
Trichophyton mentagrophytes was isolated from tinea corporis of a 56- year old male to use in experimental infection of rabbits. The isolate was diagnosed by molecular method depending on internal transcribed spacer region (ITS) with primer pairs (ITS1and ITS4) [27]. Fungal genome was extracted by using FavorPrep™ Fungi/Yeast Genomic DNA Extraction Mini Kit (Favorgen Biotech Corp., Taiwan). PCR mixture was set up in a total volume of 20 μl, including 5 μl of PCR master mix of AccuPower® PCR Premix (Bioneer, South Korea), 1μl of each primer, 1μl of template DNA and sterile deionized distilled water. Negative control containing all material except template DNA was also used. Cycling of PCR was initiated at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 35 seconds, annealing at 52°C for 30 seconds, and extension at 72°C for 60 seconds, with a final extension at 72°C for 6 min. Sequencing of PCR product was performed by Bioneer Company.
(South Korea). Fungal species was diagnosed after comparing the obtaining sequences with that recorded in GenBank by using BLAST program.

**Vaccine preparation**

Antifungal vaccine was prepared from inactivated fungal cells of isolated *T. mentagrophytes*. Approximately $8 \times 10^8$ cell/ml from old growing fungi (1 week at 30°C on Sabouraud’s Dextrose agar (Himedia, India)) was mixed with 100 ml of sterilized distilled water. The aqueous fungal suspension was heated at 70°C for 3 hours in a water bath [28]. To insure of inactivation of fungal cells, 0.1 ml of treated fungal suspension was cultured on Sabouraud’s Dextrose agar and incubated at 30°C for two weeks. A negative growth is an indicator for successful inactivation. Prepared suspension was stored at 4°C until used later as a vaccine. Freund’s adjuvant of inactive *Mycobacterium tuberculosis* was chosen as an adjuvant. It was used in two forms initiation by using a complete Freund’s adjuvant followed by incomplete Freund’s adjuvant for the remainder of time.

**Animals**

Rabbit was included in a case control study to investigate the prophylaxis and treatment ability of the dermatophytic vaccine. Ethical approval of this study was obtained from the ethical scientific committee of the College of Medicine, University of Karbala, No. 504, on 2 June 2020. A total of 18 healthy rabbits with weight 2.5 to 3.5 kg were used in this study. For the prophylaxis study, 12 healthy rabbits were divided into four groups with 3 in each. The first group was subcutaneously injected with 1 ml of vaccine only, the second with a mixture of 1 ml of vaccine and 0.1 ml of Freund’s adjuvant (Wahag Al-Dna, Baghdad), the third with 0.1 ml of Freund’s adjuvant only, and the fourth left without any vaccine or adjuvant. Groups were infected later with isolated *T. mentagrophytes* and development of dermatophytosis was followed up. Clinical changes on the infection site of all groups were observed for 6 months.

For treatment study, 6 non-vaccinated infected rabbits with *T. mentagrophytes* were divided into two groups with 3 in each. The first group was treated with 1 ml of prepared dermatophytic vaccine by subcutaneous injection once daily for 16 days. The second group, used as a control, was left without treatment. Clinical changes in infection lesions were followed up for about 3 months.

**Infection of animals**

An inoculum solution containing $8 \times 10^8$ cell/ml of *T. mentagrophytes* was prepared by mixing an amount of fungal mycelium grown on Sabouraud’s Dextrose agar (Himedia, India) for 1 week at 30°C in sterilized normal saline. Counting was performed by Haemocytometer method [29]. About 5-7 cm of the neck area of each rabbit was shaved by a mechanical method to remove covering hairs from skin. A few drops of prepared fungal suspension were inoculated on the shaved area with some pressure and spread with the fingers. Infection development was followed up for more than 3 weeks, when lesions were clinically evaluated as dermatophytosis infection.

**Statistical analysis**

Data of all tests were expressed as mean ± SD. The values were analyzed statistically with one way ANOVA by using Microsoft Windows Excel application version 10. The minimum level of $p$ value was $< 0.05$ considered as significant level.

**Results**

Rabbit was chosen to be a model to study the prophylaxis and treatment efficacy of antidermatophytic vaccine against dermatophytoses. In the prophylaxis control study, the first two groups of treated rabbits with prepared vaccine and with vaccine and Freund’s adjuvant showed resistance to infection with *T. mentagrophytes* for more
than 6 months without clinical features of infection or serious inflammation response to the adjuvant. Thus, vaccine alone showed a successful immunization effects against dermatophytosis without need of adjuvant. Meanwhile, other two groups, including those treated with Freund’s adjuvant only and without any treatment revealed clinical features of dermatophytosis after 16 days from start of infection (table 1)(fig. 1). In the second study, treatment of infected rabbits with prepared vaccine was shown to be completely curative after 8 days of treatment. The number of lesions gradually decreased after treatment with the vaccine until complete healing. In the control group, rabbits revealed no signs of cure even after 3 months (table 2)(fig. 2).

**Discussion**

Control of opportunistic fungal infections has experienced a challenge nowadays due to increase of these infections among a wide range of patients, especially those with immunocompromised conditions, cancers, a long-term treatment and in premature infants [6]. Development of resistance to antifungal agents was also associated with the difficulty in controlling them [4, 30]. Thus, a vaccine against infections by many pathogenic fungi is considered the best option to enhance the efficiency of the immune system [6, 30]. This has been taken seriously after a better understanding of the immunity toward pathogenic fungi and after increase in the incidence and mortality rate of fungal infections [6]. Moreover, successful discovery of an antifungal vaccine will play an important role to limit use of chemotherapy or antifungal agents for control of fungal infections [4]. The main effective role of antifungal vaccine in the human body is elevating the stimulation of immune system components against invasive fungi. Humoral immunity is part of the immune system most affected with the vaccine [7, 31]. This type of activation will provide protection to immunocompromised patients, especially after activation of antibody production [1]. Activation of cellular immunity represented by Th1 response with induction of IL-12 and IFN-γ is also required from an effective vaccine [30].

Antifungal vaccine is usually prepared from living or inactivated whole cells, or from one component of fungi such as cell wall components, cytoplasmic extracts, and genetic recombinant proteins [32]. Extensive researches have been performed to develop an effective vaccine against fungal infections in both man and animals [4-5]. Although some vaccines are available for use in animals, researchers are still looking for a perfect vaccine. A satisfactory result has been achieved from the vaccines used to immunize against various fungal diseases such as candidiasis, blastomycosis, coccidioidomycosis, histoplasmosis, and paracoccidioidomycosis [4]. However, the safety and application of any vaccine discovery has not yet been given a license from the US FDA for commercial use [4-5, 32].

Dermatophytosis is a typical common disease in the cutaneous layer of the skin of different parts of the human or animal body [8]. Control of this disease has been recently gained attention after elevation of drug-resistance rate in many of causative dermatophytes [33] and also because treatment of dermatophytosis, especially in animals, is usually expensive and time-consuming [7]. Thus, development of antidermatophytic vaccine can introduce a solution to decrease these disadvantages and also to limit transfer of dermatophytes between human and animals [7, 34]. However, increase of immune response against dermatophytosis can be a key to limit the toxicity and virulence effects of this disease [35]. Recently, many studies have tried to enhance a prophylactic action of the antifungal vaccine by stimulation of cellular immunity for increasing immunization rate against dermatophytosis [8]. This has been achieved by using specific antigens of a dermatophyte, especially from *Trichophyton* spp., through providing more immunization than it can gain from inactive vaccine [7-
Although no vaccine has a license for commercial use against dermatophytosis, some countries like Norway immunized their cattle by a vaccine against *Trichophyton verrucosum* as a strategy to control dermatophytosis [7]. A company Biocan M Plus in the Czech Republic also produces unlicensed vaccine of inactivated *Microsporum canis* for treatment of dogs against dermatophytosis [26].

Our prepared vaccine provided a prophylaxis to rabbits from dermatophytic infection for more than 6 months. Other studies failed to achieve this period as with the study of DeBoer and Moriello (1995) who found that dermatophytosis lesions developed in cats vaccinated with killed *M. canis* cell wall after 16 weeks of challenges with other infected cats [21]. Rabbit is often preferred for use as animal model for fungal infection over small mammals due to easy observation of changes in fungal lesions [34]. Vaccination of rabbit with culture filtrate antigens of one dermatophyte species was found to supply immunization against six other species as indicated by positive skin test [24]. Subcutaneous injection of rabbit with heat killed *Trichophyton purpureum* suspended in Freund’s adjuvant also provided protection against infection with the same fungus for more than 17 months [36]. Vaccination with heated inactivated macroconidia (6-24 x 10⁶ cell/ml) of *T. mentagrophytes* and *M. canis* protected rabbits and guinea pigs against infection with a virulent isolate of *T. mentagrophytes* [15]. Other animals have also shown resistance to infection with dermatophytes after immunization with antifungal vaccine. Vaccination of guinea pigs with *Trichophyton equinum* vaccine increased resistance to *M. canis* compared with non-vaccinated controls [24].

In this study, vaccination was performed by inactive cells of *T. mentagrophytes* either alone or with adjuvant. This type of vaccine can exhibit a better outcome in some cases than from vaccination of animals with a specific component of dermatophytes [7, 13, 18-19, 21, 25, 37]. Intramuscular injection with a vaccine of live *T. verrucosum* was found useful to protect calves from dermatophytosis [18], while purified recombinant keratinolytic metalloprotease (r-MEP3) failed to protect guinea pigs against infection with *M. canis* [13]. A preparation of live freeze-dried vaccine of *T. verrucosum* was also used successfully to protect calves against experimental dermatophytosis [25]. Vaccinated cats with killed cell wall of *M. canis* showed efficiency to stimulate production of high titer of anti-dermatophyte IgG and small cell-mediated response [21]. A vaccine of whole cells of live and killed *Aspergillus fumigatus* also provided variable protection against aspergillosis in a mouse model [37]. However, intra- or subcutaneous injection of whole cell or crude extract of dermatophytes have the ability to introduce protection in animals more than when introduced by other routes [19].

From the results of this study, there was no difference between use of prepared vaccine alone or with Freund’s adjuvant. Both vaccinated groups of rabbits were resistant to infection by *T. mentagrophytes* for more than 6 months, while rabbits treated with only Freund’s adjuvant showed infection after 16 days. This indicates that the presence of Freund’s adjuvant had no effect on the prophylaxis efficacy of prepared vaccine to stimulate the immune system. Westhoff et al. (2010) also found a similar result when they tested the prophylactic activity of non-adjuvanted inactivated vaccine in cats prepared from some strains of dermatophytes [20]. Actually, the main reason to use Freund’s adjuvant in this study was to increase a potential immunologic stimulation of prepared vaccine. Many studies investigate the efficacy of antidermatophytic vaccine in animals after mixing with adjuvant. Pier (1994) found that a suspension of killed *Trichophyton equinum* with adjuvant showed effective prophylaxis in horses and guinea pigs against infection with the same fungus or with
other species of dermatophytes [23]. Adjuvanted secreted compounds of *M. canis* with monophosphoryl lipid-A (MPLA) revealed a partial protection against infection with the same fungus in guinea pigs [14]. In general, adjuvant, which contains one or more complex compounds, is preferred for use with a vaccine of a single antigen that has a weak ability to stimulate the immune response [4, 36]. Recently, researchers have tried to develop an antifungal vaccine from purified, recombinant, or synthetic antigen, which all need adjuvant to obtain a suitable protection against infection with pathogenic fungi [1, 4, 36]. Unlike vaccine with inactivated organisms, vaccine with single antigen always has problems with their purity and production [1]. Freund’s adjuvant, which contains heat killed *Mycobacterium tuberculosis* emulsified in mineral oil, is commonly used for accelerating new vaccine testing to stimulate immunity against various infections in animal experiments [1, 4, 31]. This role can stand for a long time by lengthening fungal antigen release into the injection site [38]. Cellular immunity, such as by T-helper cells is usually elicited by Freund’s adjuvant which can also stimulate humoral immunity [1, 4, 31, 36]. However, combination of vaccine with adjuvant is used to achieve many purposes, including increased immunological response through stimulation of various immunologic pathways; alteration of immune response toward specific infection; and allowing usage of small dose of vaccine [31].

In the second part of this study, prepared antifungal vaccine showed therapeutic action against dermatophytosis in rabbits in a short time (8 days) compared with the untreated group. In another study, a filtered growth of *T. verrucosum* was prepared as injected vaccine with adjuvant for treatment of cows and buffaloes with dermatophytosis and it showed effective results after 10 days after injection [39]. In a placebo-controlled-double-blind study, mixed aqueous preparation of inactivated vaccine from six dermatophyte species exhibited better curative action for cats with dermatophytosis, especially those with first infection or at young age [17]. Inactivated vaccine of five species of dermatophytes without adjuvant was also used in another control study for treatment of cats with dermatophytosis, but without significant differences between treated cats and control group [20]. Some companies are trying to produce effective prophylaxis and treatment vaccine against dermatophytosis in animals, but their work is still not licensed by the FDA. Micanfin (Biocan-M®) vaccine manufactured by Bioveta (Czech Republic), which is composed of inactivated *M. canis*, is commercially used for the treatment of cats and dogs from dermatophytosis [16, 40]. Erman Or et al. (2005) also found that Micanfin product has therapeutic action against dermatophytosis in cats after two vaccination doses with 21 days interval [40], while another study showed that this vaccine needed 20-30 days for treatment of cats with dermatophytosis [16]. Meanwhile, Chansiripornchai and Suampairintri (2015) found that treatment of a male cat with dermatophytosis by Micanfin vaccine reduced the infectious lesions and regrowth of hair after 14 days of first injection [22]. Nedosekov et al. (2016) performed a clinical trial to evaluate the ability of the heat inactivated vaccine of *T. mentagrophytes* and *M. canis* called Funhikanifel to treat dogs and cats from experimental dermatophytosis. A single vaccination was followed by recovery of 27% of dogs, while double vaccination cured 96.8% of all animals [15].

Development of new vaccines for dermatophytosis in human has faced many challenges. The first challenge is that the majority of fungal infections affect immunocompromised patients (this is not the case with dermatophytosis) which can be resolved by choosing a vaccine with an ability to elicit humoral immunity; the second is related to the cost of vaccine preparation which has become expensive,
especially for those prepared from recombinant antigens; the third is the new vaccine may act on the normal flora in the human body when it is used against the diseases caused by one of them, for example candidiasis; use of antigen similar to that in the host that can induce unnecessary autoimmune response is the fourth challenge; the fifth one is that some types of vaccine either with or without adjuvant may not induce adequate immunization in some individuals [1, 6, 31-32, 37].

Conclusion
Although there are some studies trying to find a suitable vaccine against dermatophytosis, no one has a license from an approval organization such as FDA for use in the commercial field. A new preparation of vaccine from inactivated *T. mentagrophytes* showed effective prophylaxis and treatment results against dermatophytosis in rabbits. Long-time protection and short-time treatment are the most significant results obtained from this study.

Acknowledgment
The authors are grateful to Mr. Philip Smith for his assistance in language corrections.

Ethical statements:
Conflicts of interest: The authors have no conflict of interest to disclose
Funding: There is no funding for this research

Ethical considerations: Animal ethics were followed in this study (ethical scientific committee of the College of Medicine, University of Karbala. No. 504, on 2 June 2020).

References
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32- Hamad M. Universal fungal vaccines, could be light at the end of the tunnel?. Human Vaccines & Immunotherapeutics. 2012, 8:1758-1763.
Table (1): Infection period of vaccinated rabbits

<table>
<thead>
<tr>
<th>Group</th>
<th>Infection period (days)</th>
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<tbody>
<tr>
<td>Vaccine only</td>
<td>None*</td>
</tr>
<tr>
<td>Vaccine with adjuvant</td>
<td>None*</td>
</tr>
<tr>
<td>Adjuvant only</td>
<td>16</td>
</tr>
<tr>
<td>Control</td>
<td>16</td>
</tr>
</tbody>
</table>

*significant difference between groups at p<0.05

Table (2): Treatment period of infected rabbits after vaccination

<table>
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<th>Treatment period (days)</th>
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<td>5-20</td>
<td>8*</td>
</tr>
<tr>
<td>Control</td>
<td>6-12</td>
<td>None</td>
</tr>
</tbody>
</table>

*significant difference between groups at p<0.05

Figures legends:

Fig. 1: Infected rabbit with *T. mentagrophytes* after vaccination
A: Control rabbit with dermatophytosis lesions (red with granulated skin).
B: Vaccinated rabbit with adjuvant only which showed the same infectious features of control.
C: Vaccinated rabbit with vaccine only without any lesions of dermatophytosis for more than 6 months.
D: Vaccination with vaccine and adjuvant without any lesions of dermatophytosis for more than 6 months.

Fig. 2: Treatment of infected rabbit with inactivated vaccine.
A: Rabbit with dermatophytosis lesions before treatment.
B: Rabbit with cure of infection after 8 days of treatment.