

Dermato-kit: A newly developed dotted lateral flow immunochromatographic kit for rapid diagnosis of dermatophytosis

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Research Article

Keywords: Dermatophytosis, lateral flow, lateral immunochromatographic assay, rapid diagnostics, lab on a chip, field test, ASSURED test

Posted Date: June 8th, 2022

DOI: <https://doi.org/10.21203/rs.3.rs-1735333/v1>

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Abstract

Dermatophytosis is a widely spread contagious zoonotic disease, affecting both man (tinea) and animals (ringworm). This disease is caused by a group of closely related keratinophilic fungi known collectively as the dermatophytes group. Although the wide distribution of dermatophytosis cases throughout the whole world and its adverse clinical effect on human health, economical effect on productive animals, and pet animal welfare, there is no rapid accurate diagnostic tool for such disease. Up to date, dermatophytosis diagnosis is limited to direct, rapid with low sensitivity and specificity several wet mounts techniques, sophisticated with high skilled personnel requirement molecular assays, and gold standard with long time consumption conventional culturing and biochemical testing. All of the previously mentioned existing diagnostic methods are lab confined techniques with no availability of a field, handheld, or bedside tests. The different aspects of difficulties facing dermatophytosis diagnosis lead to mistaken diagnosis and faulty prescription of antifungals with its expected long list of side effects on man and animals. The current conducted study tries to accomplish the difficult equation by achieving an accurate, sensitive, specific, user-friendly, rapid, robust, device less, deliverable to end-users, and economic cost for development and production diagnostic kit. The newly designated and developed kit, based on lateral flow immune-chromatographic assay, showed a very promising competitive diagnostic result.

I. Introduction

Dermatophytosis is a Zoonotic disease caused by a closely related group of keratinophilic hyaline fungal agents called the dermatophytes group. It includes three main genera and more than fifty species (De Hoog HS, et al. 2017).

Dermatophytosis is a highly contagious and widely spread disease that infects both man and animal with increased and regular incidence all over the year throughout the whole world (Teklebirhan G and Bitew A. 2015).

Despite the vital need of obtaining a precise diagnosis of dermatomycoses cases, scientific funding and diagnostics investment in applied medical mycology have lagged. Public and private investment in therapeutic interventions, on the other hand, is constantly increasing. In the United Kingdom, for example, mycological diagnostic research received about 1.9 percent to 2.8 percent of the funding awarded to more than 6125 studies in infectious diseases conducted between 1997 and 2020, while clinical research in the same medical field received only 12.7 percent of this allocation, with overall 14–15 percent mycological research funding. The fraction of this small proportion of funding which included diagnostics studies in this published paper is likely to be less than a fifth of the 12.7 percent, suggesting that research in the UK, as an example of a developed country, fungal diagnostics research may receive less than 0.1 percent of government research funding, and even less in developing countries. This appears to be insufficient investment in a significant medical need. (Head MG, et al. 2014).

Direct potassium hydroxide (KOH) microscopy and fungal culture are considered the gold standard for diagnosing dermatomycosis although other molecular diagnostic techniques are existing. Direct KOH microscopic examination and other available direct microscopy-based techniques appear simple but require skill for appropriate sampling, resolving the collected skin scrapings and nail pieces, and identifying the organisms (Hassan AH, et al. 2020).

All culture-based techniques although their high specificity, all are sharing costly procedures, a long time to result, and intensive lab work (Gip L. 1981), (Banerjee U and Talwar V. 1984), (Salkin IF and Padhye AA. 1997), (Gromadzki S, et al. 2003), (Li XF, et al. 2009), (Mayser P. 2013).

Also, molecular-based diagnostic technology to amplify fungal nucleic acid with a prominent remarkable specificity and sensitivity has become a promising standard diagnostic method for laboratory diagnosis of dermatophytosis. However, it is heavily confined to a laboratory facility, and experienced staff which so, unfortunately, are not available and reachable to most public health communities and remote countryside as well as low-income countries (Begum J, et al. 2020)

With the wide impediments in the field of diagnostic mycology especially in low-income countries as the lack of specialized clinical mycologists and low cost, rapid, sensitive as well as specific diagnosis requirements, the need for a competitive diagnostic technique with sensitive, specific, and fast results obtaining and interpretation is considered a medical priority in the field of dermatophytosis diagnosis (Kozel TR and Wickes B. 2014) and (Khalil KH, et al. 2020).

ii. Materials And Method

Fungal strains obtaining

Five hot local dermatophytes strains belong to the following species: *Microsporum canis* (*M. canis*), *Microsporum gypseum* (*M. gypseum*), *Trichophyton equinum* (*T. equinum*), *Trichophyton verrucosum* (*T. verrucosum*), and *Trichophyton mentagrophytes* (*T. mentagrophytes*), were used during the current study. The used strains were isolated, identified, and selected through a previous preliminary cross-sectional study on dermatological affections of companion animals caused by dermatophytes and other keratinophilic fungi in the Greater Cairo Area, Egypt, which has been conducted by the same scientific research team (Hassan AH, et al. 2021). Those strains have been used for the preparation of antigens that were used for immunization of animals during the lateral flow kit development stages, and also were used as a positive control during the newly developed kit validation stage.

Selection of dermatophyte strains

From the isolated dermatophyte species, the most suitable strains for vaccine production were selected according to the criteria reported by (Brandebusmeyer, et.al. 1990). According to these criteria, we found that dermatophyte vaccinal strain should be:

1- Local strain.

2- Isolated from badly infected cases.

3- Rapid grower in vitro.

4-Intensive spore formers, where the dermatophyte microconidia are considered as an important character for the selection of suitable vaccinal strain. This is because microconidia have been identified to be the carrier of immunogenicity.

5- High growth intensity that allows the production of a large number of fungal antigens.

Preparation of dermatophyte antigens (Hiroaki w and Masahiro M. 2016)

Mycelial matt antigens were prepared for each dermatophyte of the five species involved in the current study as follows; The isolated, identified, and selected strains have been cultured separately on non-supplemented Sabouraud's dextrose broth, after 21 days, harvesting and separating of the superficial floating mycelial matt from the submerged soluble fungal structures using sterile gauze. Each harvested mycelial matt was homogenized using a homogenizer at 160 rpm for 5 minutes, then washed with sterile phosphate-buffered saline (PBS) three times, each time PBS suspension, centrifugation at 5000 rpm for 5 minutes, and discard of the supernatant were performed. Finally, each mycelial antigen was suspended in PBS to a concentration of 4%.

Preparation of emulsions used for animal immunization (Harold FS. 2005)

The immunization emulsion for dermatophytes mycelial antigen was prepared as follows; the priming immunization emulsion consisted of equal volumes of complete Freund's adjuvant (Sigma-Aldrich®) and inactivated previously prepared 4% mycelial antigens, inactivation was performed using 0.5% formalin (Sigma-Aldrich®), mixed adequately using two syringes and connector for 20 minutes till reached a milky white viscous creamy emulsion stable after overnight testing at refrigerator temperature (6°C). The boosting immunization emulsion consisted of equal volumes of incomplete Freund's adjuvant (Sigma-Aldrich®) and living previously prepared 4% mycelial antigens, mixed adequately using two syringes and connector for 20 minutes till reached a milky, white, viscous, and creamy emulsion with stable formulation after overnight testing at refrigerator temperature (6°C). Both types of immunization emulsions have been prepared and injected under aseptic techniques.

Lab animal selection and immunization protocol design

- **The selected lab animal (Harold FS. 2012)**

Two white male New-Zealand rabbits weighing 2 kg were designed to be used as the bio-factory for dermatophytes pAbs production. As using a fully mature animal will ensure a completely functioning immune system which is the cornerstone of antibody production. Also, rabbits were the chosen lab animal from the commonly used lab animal list used for antibody production as it is characterized by their small size, high affinity, relatively long life span, easy blood obtaining, strong immune response, and

inexpensive housing. A number of two animals were injected with the same dermatophytes Ag to enlarge the obtained blood samples carrying antibodies for each Ag, also to take in advance the unexpected accidental death of one of the two animals before the end of the injection protocol.

- The design of the animal immunization protocol (Marlies L and Coenraad FMH. 2005)

Day 0	A blood sample was collected from each animal involved in the conducted study	
Day 1	0.5ml/kg of the priming emulsion	The whole dose is injected intradermally (I/D) and divided into different 10 sites
Day 14	1ml/kg of the boosting emulsion	The whole dose is injected subcutaneously (S/C) and divided into 5 different sites
Day 28	1ml/kg of the boosting emulsion	The whole dose is injected S/C and divided into 5 different sites
Ear vein blood samples collection and Agar gel precipitation tests were performed		
Day 38	1ml/kg of the boosting emulsion	The whole dose is injected S/C and divided into 5 different sites
Ear vein blood samples collection and Agar gel precipitation tests were performed		
Day 48	1ml/kg of the boosting emulsion	The whole dose is injected S/C and divided into 5 different sites
Ear vein blood samples collection and Agar gel precipitation tests were performed		
Day 55	1ml/kg of the boosting emulsion	Half of the dose S/C is divided into 2 different sites and the other half of I/M is divided into 2 different sites
Ear vein blood samples collection and Agar gel precipitation tests were performed		
Day 62	1ml/kg of the boosting emulsion	Half of the dose is injected S/C and divided into 2 different sites and the other half is injected intramuscularly (I/M) and divided into 2 different sites
Ear vein blood samples collection and Agar gel precipitation tests were performed		
Day 79	1ml/kg of the boosting emulsion	Half of the dose is injected S/C and divided into 2 different sites and the other half is injected I/M and divided into 2 different sites
Ear vein blood samples collection and Agar gel precipitation tests were performed		
Day 90	Final blood collection	

Table (1): Illustration of a detailed animal immunization schedule**Separation and purification of polyclonal antibodies (PAbs) (Federico P, et al. 1990)**

The separately collected blood samples were kept for 1 hour undisturbed in a slightly oblique position at room temperature (25°C), followed by another hour in the refrigerator (6°C). Then 25ml of blood was centrifuged for 30 minutes at 10000 × g, the supernatant was collected, and the pellet was discarded. The obtained serum was placed on a magnetic stirrer, then dropwise of caprylic acid, 2.02 ml/25ml rabbit serum was dropped slowly while stirring at 25°C for 30 min. The mixtures were centrifuged at 10000 × g for twenty min, the supernatants were collected, and the pellets were discarded. The collected supernatants were dialyzed separately against PBS at 4°C overnight using 12000–14000 molecular weight cut off (MOWC) dialysis bags (Sigma-Aldrich®) with three buffer changes, finally, the concentration of immunoglobulins was obtained by measuring both the total protein and the albumin concentrations of each obtained purified antibodies sample from the dialysis bags. The obtained immunoglobulins concentrations ranged from 1.1 to 1.4 g/dl. A 1 mg/ml concentration was then obtained using ultrapure water dilution. Furtherly, half the volume of the separately obtained dermatophytes species-specific polyclonal antibodies were pooled together in equal ratios to form a dermatophyte multispecies-specific polyclonal antibody (PAbs).

Agar gel precipitation test optimization for evaluating the separated PAbs (Rowa YA. 2018)

Agar gel plates were prepared as follows; 3mm agar gel thickness and 0.6% concentration were achieved by dissolving 0.6 g agarose in 100 ml PBS, then dissolved by boiling for 2 minutes and pouring 4ml from the preparation in 5 cm plates, then left to solidify. The agar gel test was used in several situations during the current study; First, to monitor the antibody production during the designed immunization protocol, second, to evaluate the consequence of the PAbs purification steps, third, to study the relationship between the produced species-specific PAbs and the prepared immunizing antigens, which derived from different dermatophyte species and the relationship between the produced dermatophyte multi-specific PAbs and other non-dermatophyte keratinophilic fungal antigens.

Preparation of colloidal gold (CG) nanoparticles (NPs) (Ying W, et al. 2014), (Lev D and Nicoli G. 2019), and (Petr S, et al. 2020)

50 ml of ultrapure water were placed on a hot plate till boiling (100°C), then vigorous magnetic stirring was started followed by the addition of 0.5ml of 0.2% HauCl₄.3H₂O (Gold III chloride trihydrate) (Sigma-Aldrich®). During the boiling of the mixture with vigorous stirring, 1 ml of 1% (w/v) sodium citrated buffer was added quickly. The color of the mixture began as a colorless solution changed to black then ruby red color (after approximately 2 minutes), then kept on stirring and at the boiling temperature (100°C) for another 10 min. The prepared nanoparticles were scanned at a range of 400–600 nm using a UV-Vis spectrophotometer and Transmissible electron microscope (TEM) imaging to determine their diameter. The obtained colloidal gold nanoparticles were confirmed to be 40 nm in diameter

Conjugation of the purified rabbit polyclonal antibodies with the colloidal gold nanoparticles (Au NPs-PAbs coupled bio-conjugate) (Constance O, 2010), (Mir HJ, et al. 2016), (Sadaki Y, 2016)

The performance of any newly developed lateral immunochromatography-based kit mainly depends on a variety of parameters, such as the antibody labeling by the gold nanoparticles, the pH value set within the strip microenvironment, and at which the Ag-Ab immune-complex is expected to occur if the target analyte exists, amount of specific antibodies required for optimal immunological coupling, and finally the antigen/antibody loading concentration of the test/control spots. Based on the colloidal gold preparation methodologies, the most stable and unaggregated immune-gold bio-conjugate is achievable at a pH that is slightly higher or near the isoelectric point of the conjugated protein. The highest adsorption capacity of antibodies on the surface of the gold nanoparticles was achieved by fixed unchanged ruby red color formed in the pH series between 7.2–7.6. 0.02 M potassium carbonate (K₂CO₃) was used to adjust the pH of colloidal gold to 7.4. The amount of antibodies that can form stable conjugates and optimally reacted test and control spots is another key factor for the success of the newly developed kit. To determine the optimal amount of antibodies, different concentrations of anti-dermatophytes IgG were tested. However, Au NP solution (100 µL) containing 1.0 µg of PAbs of anti-dermatophytes IgG on the conjugate pad could remain as stable ruby red reactions after exposure to the positive and negative control solutions. Half ml of purified multi-species rabbit PAbs (1 mg/ml) was added to 50 ml of colloidal gold nanoparticles. The solution was gently mixed for 10 minutes. Then centrifuged for 10 min at 10.000 × g, discarding the supernatant and keeping the pellet. The conjugated pellet was diluted in 1 ml conjugation dilution buffer, which was prepared as follows; 20 mM Tris, 0.02%(w/v) sodium azide, 3% (w/v) sucrose, and 1% (w/v) bovine serum albumin (BSA)) and stored in a refrigerator (6°C).

An overall newly developed kit fabrication and formulation procedures (Claudio P, et al. 2020)

- **Preparation of the lateral flow solid phase (multi-laminated membranes strip) (Mansfield, et al. 2005)**

Sample pad roll (AhIstrom®)

pre-treated glass fiber with sample pad treatment solution pH 7.4 (ultra-pure water, 2% (w/v) triton X-100, 3.81% (w/v) Borax, 1% (w/v) Polyvinylpyrrolidone (PVP), 0.5% (w/v) sodium cholate, 0.15% (w/v) sodium dodecyl sulfate (SDS), 0.1% (w/v) casein sodium salt, 0.02% (w/v) sodium azide, then dried for 1hr at 37°C.

Conjugation pad roll (AhIstrom®)

pre-treated glass fiber with conjugation treatment solution pH 7.4 (2.5% (w/v) sucrose, 20 mM PBS, 0.3% (w/v) PVP, 2% (w/v) BSA, 1% (w/v) triton X-100 and 0.02% (w/v) sodium azide).

Nitrocellulose membrane (NC) roll (AhIstrom®): Microporous nitrocellulose membranes are used as the carrying matrix upon which Ag-Ab immunocomplexes are formed and visualized to indicate the presence or absence of the target analytic in liquid samples. Several reasons contributed to making NC the preferred LF solid phase: High affinity of protein adsorption, the chemical properties that led to stable

protein adsorption levels in both dry and wet conditions of the NC membrane, and nitrocellulose membranes can be cast that have pores sufficiently large to allow a lateral flow of fluid in a reasonable time.

Absorbent (wick) pad roll (Ahlstrom®)

a cotton fiber pad roll, whose function is to collect the liquid processed through the strip by capillary action, offering consistent absorbency for test reproducibility, also allowing quick clearing of the membrane by absorbing higher volumes of liquid and access to the read-out in a shorter time.

Adhesive Polyvinyl chloride (PVC) sheets (Ahlstrom®)

A four-row laminated adhesive membrane as the carrier base of the rest components of the lateral flow strip, starting with removing the sticker from the second row and attaching the nitrocellulose membrane, followed by removing the sticker from the first row and attaching the absorbent pad, the third step was removing the sticker from the third row and attaching the conjugate pad, finally removing the sticker from the fourth row and attaching the sample pad which is diagrammatically illustrated in (Fig. 1).

Figure (1)

diagrammatic illustration of the main steps to build up the laminated lateral flow solid phase

Figure (2)

diagrammatic illustration of the building structures of each single lateral flow strip

- Loading of the different bio-reactants on the prepared lateral flow solid phase**

To yield clear and accurate results, coating anti-dermatophytes PAbs and goat anti-rabbit IgG PAbs were optimized. The red on the C line was visible when the concentration of goat anti-rabbit IgG was more than 0.3 mg/mL and became clearest as the coating concentration increased to 0.5mg/ml. As for the T line, the color spot and uniform distribution were observed when the coating concentration was more than 0.5 mg/ml. To meet the requirements of the test strip observation, 1 mg/ml of anti-dermatophytes PAbs and 0.5 mg/ml of the polyclonal goat anti-rabbit IgG antibody (pH 7.4) were chosen as the optimal concentration. The antibody printer (IsoFlow®) was used to load the 0.5 μ l of unlabeled goat anti-rabbit antibodies in the form of dots on the control spots, while the test spots were loaded as follows:

- Single-plex edition:** This is a single test spot dotted edition, which was developed in 6 versions; 1 dermatophyte multi-species version that was dotted with 1 μ l of dermatophyte multi-species PAbs at the test spot and 5 dermatophytes species-specific versions dotted with 1 μ l of dermatophyte species-specific PAbs at the test spot.
- Multiplex edition:** This is a multi-test spot dotted edition

The space between the test spot and control spot was adjusted to be 0.5 cm in the single-plex edition. The conjugate pad was loaded by impregnation in the conjugate solution achieving 5 µl conjugate on each cut conjugate pad of each strip.

- **Cutting of the test strips and packaging in lateral flow cartilage and the kit assembly**

The loaded adhered to the PVC card developed lateral flow sheets, automatically were cut by automatic Guillotine cutter achieving 0.4 cm width per strip, then due to the research level production quantity, manual assembly, and packaging of the strips into the lateral flow cartilage has occurred. The used cartilage is characterized by (S) letter labeled sample application window and (T and C) letter labeled window marking the test and control spots on the strip. The kit was assembled as a full set containing, the LFIA cartilage/cassette, scrapping brush, sample collection card, antigen extraction solution, and sample application pipette.

Preparation of antigen extraction solution

Due to the solid nature of the dermatological affection samples (hair, skin scraps, scales, nails), a “pre-application sample processing step” must be performed to extract the dermatophytes’ antigenic structures from the collected sample. Based on several laboratory-based trials and previously published literature (Hiroaki w and Masahiro M. 2016), (Noriki S. 2013), (Noriki S. 2015), (Higashi Y, et al. 2012), (Tsunemi Y, et al. 2014), (Tsunemi Y, et al. 2015) this solution was formulated and prepared as follows: 5 ml glycerin, 1ml propylene glycol, 1 g sodium dodecyl sulfate (SDS), 1 g dimethyl sulfoxide (DMSO), and 0.2 g Diazolidinyl urea were stirred and dissolved in 94 ml distilled water.

Collection of the samples used during the developed lateral flow testing and challenge with the wet mounts and cultures

A total of 100 samples (Skin scraps, scales, and hair) were collected from a wide range of companion animal species; Dogs, cats, horses, donkeys, cows, buffalos, rabbits, rats, and guinea pigs. All samples have been collected from animals suffering from dermatological affections and suspected by their clinician it would be a dermatophytes infection. All samples have been collected nearly by the same standardized sampling protocol by the hands of expert veterinarians and submitted either to LeptoVet® (an ISO 9001 accredited and licensed veterinary laboratory in Egypt) or to the mycology unit at the department of microbiology, faculty of veterinary medicine, Cairo University. These samples were directly tested through wet mounts and then confirmed as positive active dermatophytosis cases or negative cases by the gold standard selective dermatophytes culturing techniques (Hassan AH, *et al.* 2021).

Direct microscopic examination of the collected samples (Quinn, *et al.* 1990) and (Haldane DJ and Robart E. 1990)

Few hairs, and/or skin scraping were placed on a sterile slide. Few drops of potassium hydroxide (KOH) 10% with dimethyl sulfoxide (DMSO, Serva®) and glycerin (Serva®) were added to the sample (DMSO enhances KOH 10% clarifying ability, and glycerin as a source of humidity reducing the required

incubation time). The slide was covered with a coverslip, heated gently, left in a humid chamber for 30 minutes, and examined using low and high dry power lenses of a bright-field microscope (Olympus CX31 microscope with camera model C-7070).

Culturing of the collected samples (Quinn, *et al.* 1990) and (Hassan AH, *et al.* 2021)

A total of 100 samples were examined by culturing on Sabouraud's dextrose agar (SDA, Oxoid®) supplemented with 0.5 g/L chloramphenicol (Oxoid®) and 0.5 g/L cycloheximide (Oxoid®). Each sample was inoculated in 3 sets of supplemented SDA slopes and each set consisted of 3 slopes. The first set was only supplemented with the basic selective supplements (Chloramphenicol and cycloheximide) the second set was extra supplemented with specific supplements (thiamine (Serva®) and inositol (Serva®)) and the third set extra supplemented with different specific supplements, (Nicotinic acid (Serva®)). The first and third sets were incubated at 25°C and observed for up to 21 days while the second set was incubated at 30°C and observed for up to 30 days.

The newly developed kit testing procedures

Brushing the active peripheral border of the suspected lesion using the scrapping brush. The sample was collected in a black collection card to assess the amount of the collected sample. Then the sample was added to a 0.5 ml extraction solution with gentle mixing for 5 minutes at room temperature (25°C). 100 µl (3 drops) of the extracted antigen was applied to the sample application window using the application pipette. The presence of dermatophyte antigens was detected by the appearance of a specific color tracer of Au NPs.

Figure (3)

the newly developed Dermato-kit components

iii. Results And Discussion

Agar gel precipitation test (AGPT)

1. During the immunization protocol, serum was tested against the prepared antigens by AGPT, it showed no results after the 3rd injection, a weak result after the 5th injection while strong clear defined results started after the 8th injection.
2. After the purification of PAbs, purified PAbs were tested against the prepared antigens, AGPT was still showing strong results as a confirmatory step for the success of the PAbs separation and purification with caprylic acid.
3. Before the involvement of the purified PAbs in the LFIA preparation, the purified dermatophyte species-specific PAbs were tested against:
 - The prepared different dermatophyte antigens for antigenic relationship determination, and no antigenic relationships were found between the different dermatophyte species prepared antigens.

- Other non-dermatophyte keratinophilic fungal antigens prepared from the following genera and species (*Candida albicans*, *Candida tropicalis*, *Candida globrata*, *Candida krusei*, *Malassezia pachydermatis*, *Aspergillus niger*, *Aspergillus fumigatus*, *Aspergillus flavus*, *Fusarium oxysporum*, *Fusarium chlamydosporum*, and *Alternaria alternate*), also no antigenic relationships were found between dermatophyte antigens and other non-dermatophyte keratinophilic fungal antigens.

Specificity and stability of the newly developed LFIA strips

The specificity of our LFAs was tested by employing cutaneous samples including samples of clinically diagnosed cases of dermatophytosis, with alopecia, itching, and skin crusting. Each sample was tested in duplicates at two weeks without any changes, demonstrating the excellent stability of the LFIA strips produced. Further prospective studies are needed for further investigation of cross-reactivity with other cutaneous affection causes.

Principle of the developed LFIA strip

Direct antigen-antibody interaction was demonstrated to form an Antigen-Antibody-Au NPs-PAbs complex for the detection of dermatophyte antigens presence in hair, nail, and skin scrap samples. An appropriate amount of sample was added to the antigen extraction solution; the sample then was applied in the sample application window in the LFIA cartilage. The sample moved forward along the strip under the capillary effect. If the sample contains target dermatophyte antigens, the antigens will bind to the labeled rabbit anti-dermatophytes IgG-PAbs present in the conjugate pad of the cassette, and the complex would migrate to the membrane-bound unlabeled anti-dermatophytes IgG on the test spot turning it to ruby red, showed that the sample was positive. On the other hand, the unbound conjugates continued to flow forward and bound to unlabeled goat anti-rabbit PAbs (Invitrogen-Thermo-fisher®) on the control spot, turning it ruby red also. If the sample does not contain the target dermatophyte antigens, only the control spot will turn ruby red. Therefore, the LFAs are positive if two ruby red spots appeared on both test and control spots while it was negative when only one ruby red dot appeared at the control spot. Invalid strips showed no ruby red dots either in the test and control spots or showed only one ruby red dot in the test spot as illustrated in **Figure (4 and 5)**.

Figure (4): diagrammatic illustration of the working principle of the newly developed kit

Figure (5)

illustration of the valid (positive/negative) and the invalid visible outcome results

Figure (6)

demonstration of the negative reaction appearance in the newly developed kit

Figure (7)

demonstration of the positive reaction appearance in the newly developed kit

Testing of clinical dermatological samples by the LFIA strips

Clinical practicability of the LFIA was evaluated through dermatological sample testing to detect cutaneous samples collected from 100 skin affections suffered cases a few days (1–3 days) after their clinical diagnosis, where culture-negative confirmed cutaneous samples were used as the negative control. All KOH direct microscopic examination, standard cultures, and LFIA results are summarized in **Tables (2, 3, and 4)**.

Direct microscopic examination (DME)		
	Results	Positive
LFIA	Positive	6
	Negative	82

Table (2)

comparing LFIA and microscopic slide results

LFIA		
	Results	Positive
Culture	Positive	14
	Negative	79

Table (3)

comparing culture and LFIA results

Culture		
	Results	Positive
DME	Positive	5
	Negative	76

Table (4)

comparing microscopic slide and culture results

The specificity and sensitivity of the newly developed kit were calculated as follows;

$$\text{Specificity} = T- / ((T-) + (F+)) \times 100\%$$

$$\text{Sensitivity} = T+ / ((T+) + (F-)) \times 100\%$$

The LFAs gave a specificity of 98% and a sensitivity of 76.9%. The Cohen's kappa statistic was calculated to measure the level of agreement between the newly developed kit and the standard culture as follows;

Cohen's kappa coefficient (k) = $(p_o - p_e) / (1 - p_e)$, where (p_o) is the relative observed agreement among raters and (p_e) is the hypothetical probability of chance agreement

The result was 0.76 interpreted as a "substantial agreement" with the standard culture. Although the LFAs have many advantages, after all, LFA is usually only used to acquire a preliminary diagnosis. With the evaluation of the performance, the current study is helpful to explore future diagnostic applications in the LFA field. A possible improvement strategy focusing on identifying new signal amplification strategies and quantification systems warrants further study. The lateral flow-based techniques are considered a promising competitor to both conventional laboratory-based diagnostic methods and advanced molecular-based diagnostic techniques. Enhancing the lateral flows' diagnostic accuracy, sensitivity, and specificity is the recent challenge and prospect for dermatophytosis diagnosis.

Iv. Conclusion

To conclude, the conducted study described the development, optimization, and validation of a new point-of-care (POC) and field test based on the specific interactions between dermatophyte antigens and dermatophyte antigens specific antibodies. Achieving an accurate diagnosis by detecting the dermatophyte antigens in hair, nail, and skin scrap samples from dermatologically affected animals as a substitution to the most commonly used and rapid, however imperfect direct wet prepared slides and the time consuming expensive specific culturing. The newly developed lateral flow immunochromatographic assays (LFAs) can potentially provide a preliminary and competitive test result for clinicians to make the appropriate diagnosis and provide initial proper treatment to affected cases along with alternative testing methods and clinical findings. Moreover, applicability of the new LFAs is faster and easier than other methods such as direct wet mounts, implementation of isolation, and molecular techniques, especially in most medical facilities as well as remote countryside and low-income countries or regions. With the widespread nature of dermatophyte infections, it becomes an essential demand to achieve an accurate, rapid, specific, and sensitive diagnosis for dermatophytosis. On this occasion, the current serological LFAs are supposed to be a more efficient and cost-effective solution to deal with the dermatophytic affections.

Declarations

Acknowledgments

We thank Prof/Dr. Rafik Tawfik Soliman, professor of microbiology, faculty of veterinary medicine, Cairo University, for his help, advice, and support through the different stages of the conducted study. We want

to acknowledge Prof/Dr. Ahmed Samir, professor of microbiology, faculty of veterinary medicine, Cairo University, for his collaboration during sample collection stages throughout the entire study.

Ethical statement

The guidelines of the institutional animal care and use committee (IACUC) were completely followed during any procedures involving animal use through the current conducted study. And the IACUC approval and oversight on this study was obtained.

Author contributions

Heidy Abo-Elyazeed: Lead, coordinated, and designed the flow of the work and writing reviser and editor of the collected data. Hassan Aboul-Ella and Rafik Sayed: development, preparation, and evaluation of the lateral flow strips and analysis as well as interpretation of the obtained data.

Declaration of conflicting interests

The authors declared no potential conflicts of interest concerning the research, authorship, and/or publication of this article.

Funding

This study was self-funded.

Supplementary Material

Supplementary material for this article is available online at https://www.youtube.com/playlist?list=PL1uGfMXH_u0uNsBrNaFlaD82OcXouiq10.

References

1. Banerjee U, Talwar V. Evaluation of modified dermatophyte test medium for isolation of dermatophytes: A preliminary report: bewertung eines modifizierten Dermatophyten-Testmediums zur Isolierung von Dermatophyten Eine vorläufige Mitteilung. Mycoses. 1984;27(9):465–469.
2. Brandebusmeyer, E., 1990. Studies of virulence, tolerability and efficacy of a vaccine against ringworm in cattle to cattle and guinea pigs. Diss.Tierarztl. Hochsch. Hanover.
3. Begum J, Mir NA, Lingaraju MC, Buyamayum B, Dev K. Recent advances in the diagnosis of dermatophytosis. J Basic Microbiol. 2020 Apr;60(4):293-303. DOI: 10.1002/jobm.201900675. Epub 2020 Jan 31.
4. Constance Oliver. Conjugation of colloidal gold to proteins. Methods Mol Biol.2010;588:369-73.doi:10.1007/978-1-59745-324-0_39.

5. Claudio P et al. Tutorial: design and fabrication of nanoparticle-based lateral-flow immunoassays. *Nature protocol.* 2020; DOI:10.1038/s41596-020-0357-x.
6. De Hoog HS, Dukik K, Monod M, et al. Toward a novel multilocus phylogenetic taxonomy for the dermatophytes. *Mycopathologia.* 2017;182(1–2):5–31.
7. Federico Perosa, Raffaella Carbone, Soldano Ferrone, and Franco Dammacco. Purification of human immunoglobulins by sequential precipitation with caprylic acid and ammonium sulphate. *Journal of immunological methods.* 1990; 128(1):9-16.
8. Gip L. A rapid screening method for the detection of pathogenic fungi on the skin. *Mycoses.* 1981;24(1):17–26.
9. Gromadzki S, Ramani R, Chaturvedi V. Evaluation of a new medium for identification of dermatophytes and primary dimorphic pathogens. *J Clin Microbiol.* 2003;41(1):467–468.
10. Haldane DJ, Robart E. A comparison of calcofluor white, potassium hydroxide, and culture for the laboratory diagnosis of superficial fungal infection. *Diagn Microbiol Infect Dis.* 1990;13(4):337–339.
11. Harold F. Stills, Jr., Adjuvants and Antibody Production: Dispelling the Myths Associated with Freund's Complete and Other Adjuvants, *ILAR Journal*, Volume 46, Issue 3, 2005, Pages 280–293, doi.org/10.1093/ilar.46.3.280.
12. Harold F. Stills. Chapter 11 - Polyclonal Antibody Production, In American College of Laboratory Animal Medicine, The Laboratory Rabbit, Guinea Pig, Hamster, and Other Rodents, Academic Press, 2012, Pages 259-274.
13. Higashi Y, Miyoshi H, Takeda K et al. Evaluation of a newly-developed immunochromatography strip test for diagnosing dermatophytosis. *Int J Dermatol* 2012; 51: 405–409.22.
14. Head, M.G.; Fitchett, J.R.; Atun, R.; May, R.C. Systematic analysis of funding awarded for mycology research to institutions in the UK, 1997–2010. *BMJ Open* 2014, 4, e004129.
15. Hiroaki wakamoto and Masahiro Malyamoto. Development of a new dermatophytes detection device using immunochromatography. *J Med Diagn Meth* 2016, 5:2. DOI:10.4172/2168-9784.1000216.
16. Hassan AH, Rafik Hamed, Heidy Abo Elyazeed. Recent trends in rapid diagnostic techniques for dermatophytosis. *International Journal of veterinary science and medicine.* 2020.
17. Hassan AH, Rafik Hamed, Heidy Abo Elyazeed. Cross-sectional study on dermatological affections of companion animals caused by dermatophytes and other keratinophilic fungi in Greater Cairo Area, Egypt. 2021; *Advances in animal and veterinary sciences.* 9:615-622.
18. Kozel TR, Wickes B. Fungal diagnostics. *Cold Spring Harb Perspect Med.* 2014;4(4):a019299. Published 2014 Apr 1. doi:10.1101/csfperspect.a019299
19. Khalil KH, et al. Biosensors and Diagnostics for Fungal Detection. *Journal of fungi.* 2020.
20. Li X-F, Shen Y-N, Chen W, et al. A new medium for diagnosis of dermatophyte infection. *Eur J Dermatol.* 2009;19(1):34–37.
21. Lev D, Nicoli G. Methods for chemical synthesis of colloidal gold. *Russian Chemical Reviews.* 2019; 88.

22. Marlies L, Coenraad FMH. Critical Steps in the Production of Polyclonal and Monoclonal Antibodies: Evaluation and Recommendations. *ILAR journal*. 2005; 46:269–279.
23. Mayser P. A simple and rapid method to differentiate *Arthroderma benhamiae* from *Microsporum canis*. *J Dtsch Dermatol Ges*. 2013;11(4):322–327.
24. Mir HJ, et al. Various methods of gold nanoparticles (GNPs) conjugation to antibodies. *Sensing and biosensing research*. 2016; 9:17-22.
25. Noriki S. Non-heating detection method for dermatophyte. Japanese Patent No. 5,167,488. B2 (11 January, 2013).
26. Noriki S. Non-heating detection method for dermatophyte. United States Patent No.8,962,264. B1 (24 February, 2015).
27. Petr S, et al. Methods of Gold and Silver Nanoparticles Preparation. *Materials*. 2020; 13.
28. Quinn PJ. Clinical Veterinary Microbiology, section 3: Mycology, The Dermatophytes. Wolfe publishing. 1990. 67: 381-390.
29. Rowa YA. Precipitation and Agglutination Reactions; Basic serological testing. 2018. 569:95–103.
30. Salkin IF, Padhye AA. A new medium for the presumptive identification of dermatophytes. *J Clin Microbiol*. 1997;35(10):2660–2662.
31. Sadaki Yokota. Preparation of Colloidal Gold Particles and Conjugation to Protein A/G/L, IgG, F(ab')2, and Streptavidin. *Methods Mol Biol*, 2016;1474:61-71. DOI: 10.1007/978-1-4939-6352-2_4.
32. Tsunemi Y, Takehara K, Miura Y et al. Screening for tinea unguium by Dermatophyte Test Strip. *Br J Dermatol* 2014; 170:328–331.23.
33. Tsunemi Y, Takehara K, Miura Y et al. Diagnosis of tinea pedis by the Dermatophyte Test Strip. *Br J Dermatol* 2015; 173: 1323–1324.
34. Teklebirhan G, Bitew A. Prevalence of dermatophytes infection and the spectrum of dermatophytes in patients attending a tertiary hospital in Addis Ababa, Ethiopia. *Int J Microbiol*. 2015;2015:653419.
35. Ying W, et al. Preparation of Colloidal Gold Immunochromatographic Strip for Detection of Paragonimiasis skrjabini. *PLoS ONE* 9(3): e92034. <https://doi.org/10.1371/journal.pone.0092034>.

Figures

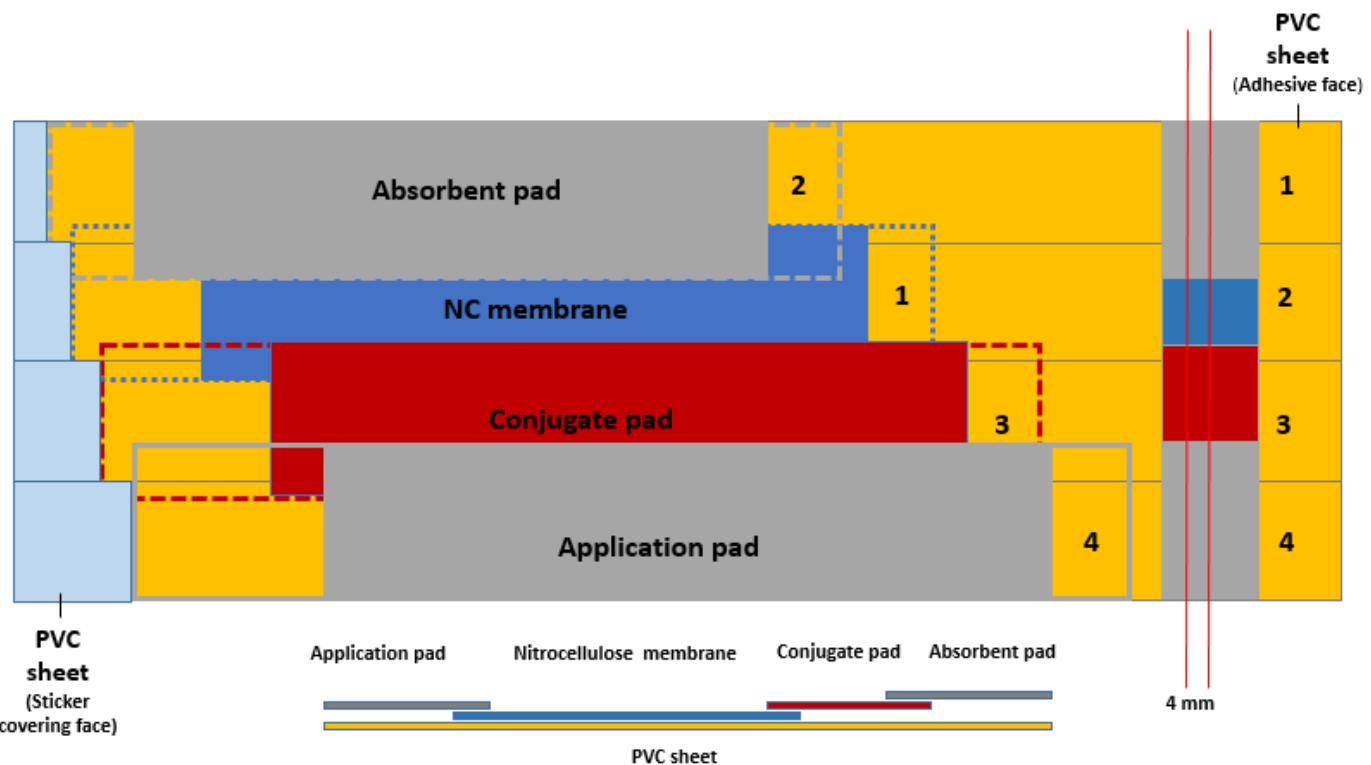


Figure 1

diagrammatic illustration of the main steps to build up the laminated lateral flow solid phase

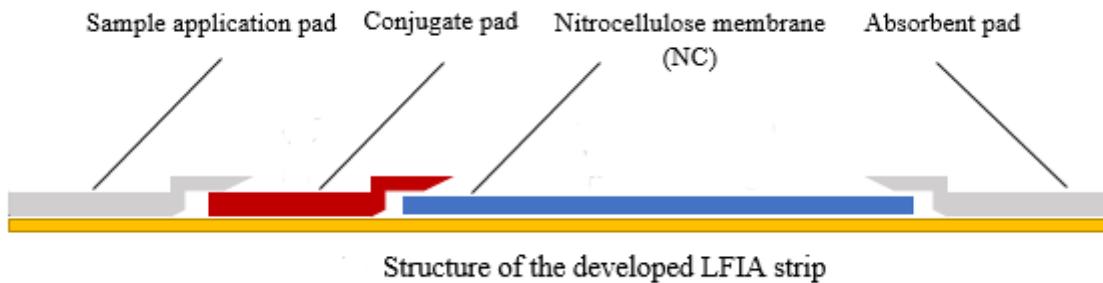


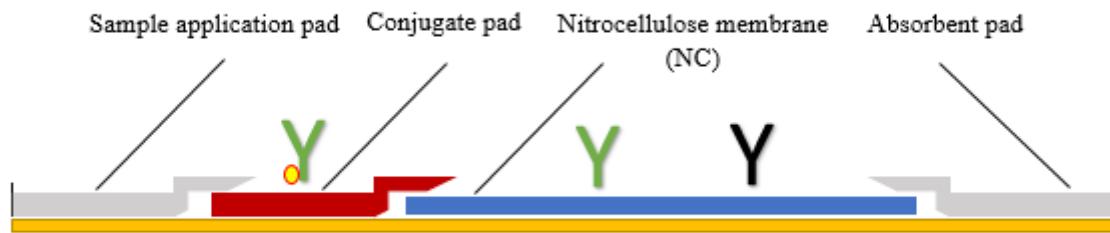
Figure 2

diagrammatic illustration of the building structures of each single lateral flow strip

Sample collection card

Figure 3

the newly developed Dermato-kit components



A) Structure of the developed LFIA strip



B) Positive detection of dermatophyte antigens



C) Negative detection of dermatophyte antigens

Dermatophyte antigens in the sample

Nano-gold labelled Ag-specific rabbit antibodies

Unlabeled Ag-specific rabbit antibodies

Goat anti-rabbit antibodies

Figure 4

diagrammatic illustration of the working principle of the newly developed kit

Figure 5

illustration of the valid (positive/negative) and the invalid visible outcome results



Figure 6

demonstration of the negative reaction appearance in the newly developed kit

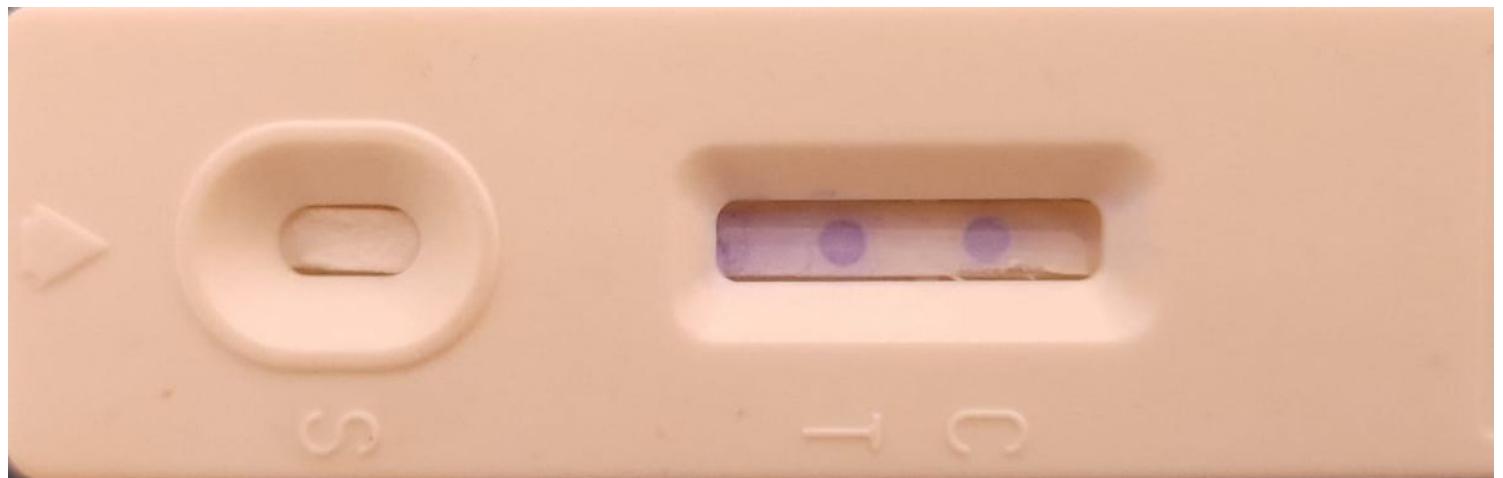


Figure 7

demonstration of the positive reaction appearance in the newly developed kit