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Evaluation of a New Multiplex Lateral Flow Immuno-Assay (LFIA)-Based Point-of-Care Test (POCT) for Rapid Diagnosis of Invasive *Fungal Infections* (IFIS) in Field-Level

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BACKGROUND

Respiratory health has become a major concern for many of the 2.77 million service members who have served in Iraq and Afghanistan. Many Veterans who served in these countries have developed acute respiratory infection (ARI) due to airborne hazards like dust storms, explosions, fumes from aircraft exhaust, and exposure to infectious agents. Fungal infections are established causes of ARI in the military during field deployment. Nevertheless, fungal infections such as coccidioidomycosis, aspergillosis, and cryptococcosis are infrequently considered or tested by clinicians who care for ARI, resulting in delayed diagnosis and appropriate treatment. Here, we report the development of a new multiplex Lateral Flow Immuno-Assay (LFIA) for the detection of serum antibodies against Aspergillus, Cryptococcus, and Coccidioides in one test format. Since most fungi possess a robust cell wall, efficient isolation of pure proteins has become a challenge in developing diagnostic assays. To circumvent this bottleneck of fungal proteins, we identified serological immunodominant peptides for *Coccidioides immitis*, *Aspergillus fumigatus*, and *Cryptococcus neoformans* which elicit antibodies during infection in humans. In a Proof-of-Concept study, the antigenicity of the immunodominant peptides was assessed in mice and the antisera was tested in ELISA and Western blot. A prototype format of indirect sandwich LFIA was developed using mice antisera as a source of the primary antibody and biotinylated immunodominant peptides immobilized on a nitrocellulose membrane in the presence of streptavidin as capture reagents. A positive result was triggered when the immuno-complexes were stopped by the individual immunodominant biotinylated peptide after 5 minutes. The multiplex LFIA developed in this study will be a valuable tool in the rapid diagnosis of fungal infections in military service members pending validation in clinical samples.

METHOD (S)

1 Synthesis of Immunodominant Fungus Peptides

Based on immune response we rationally selected and synthesized conserved immunodominant peptides from the consensus amino acid sequences of *Aspergillus fumigatus*, *Cryptococcus neoformans* and *Coccidioides immitis* based on host immune response. In terms of conjugating the peptides to the carrier protein KLH for mice antibody generation, we coupled cysteine (C) residue to C-terminal region of each peptide.

2 Generation of Mouse Anti-peptide Antibodies

Three groups of female Balb/c mice (6-8 weeks old) were immunized using 50ug KLH conjugated peptides emulsified with Freund's Complete Adjuvant (FCA). Boost immunizations using Freund's Incomplete Adjuvant (FIA) with all three peptide conjugates successfully steered immune responses toward the elicitation of precise antibody specificities to the whole fungus cell surface proteins (isolated from culture), which were confirmed by conventional ELISA and Western blot assays.

3 Mouse Anti-peptide Antibody Titer Analysis by ELISA

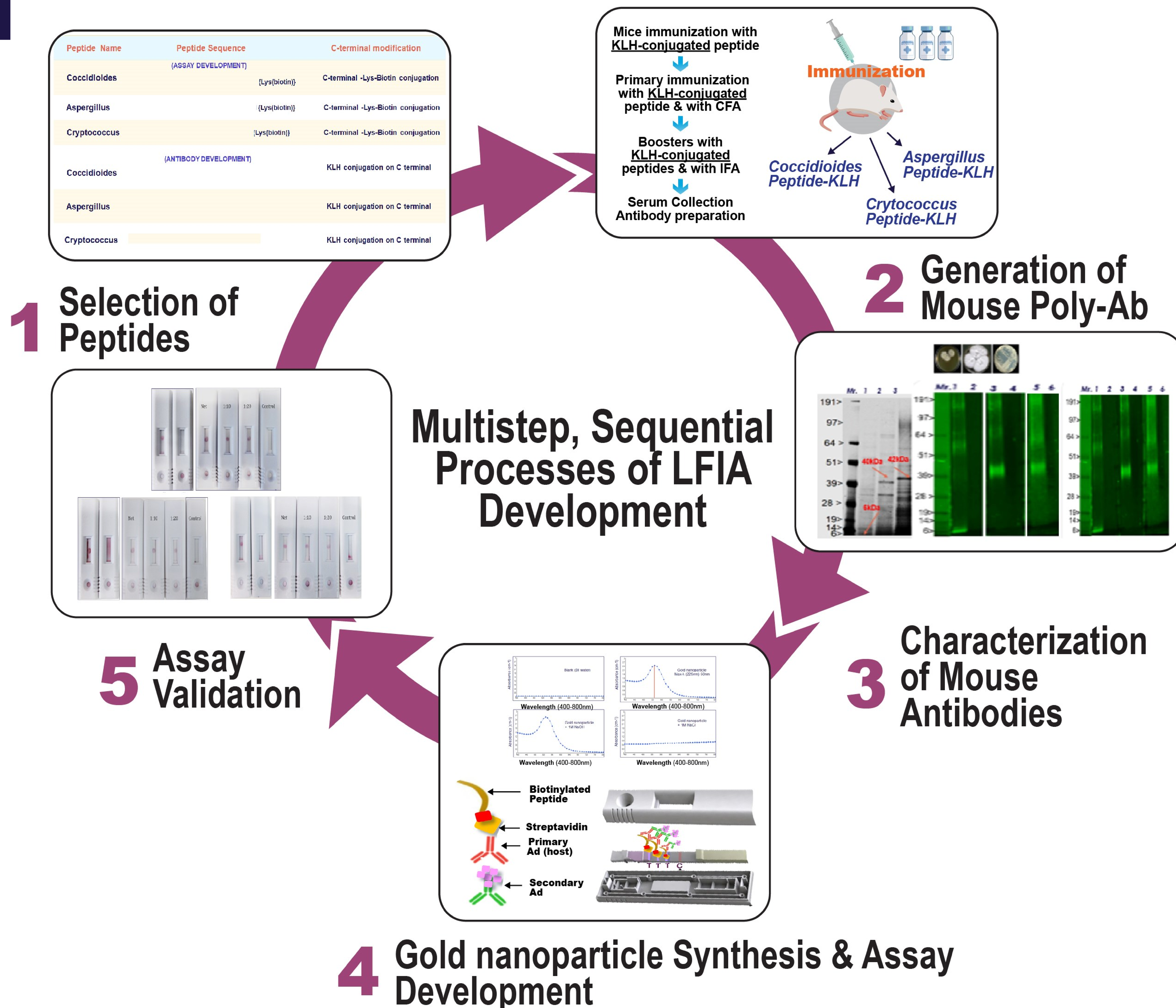
A 96 well ELISA plate was coated overnight at 4^oC with 100µl/well of cell surface proteins (20µg/ml) diluted in 0.05 M carbonated buffer (pH 9.6). The wells were blocked with 5% milk protein. After blocking, different dilutions of mouse serum samples along with control serum were added in duplicate and the plate was incubated at RT for 1hr. The plate was washed with PBS-T, and bound antibodies detected by HRP-conjugated Goat anti-mouse IgG (H+L) at 1:1K, followed by KPL SureBlue TMB substrate. The enzyme-substrate reaction was stopped by adding 1N HCl. The plate was read at 450nm in a Spectra Max M5e plate reader.

4 SDS-PAGE and Western Blot

SDS-PAGE and Western Blot were performed using lysates from cultured fungal cells with appropriate negative control. Protein samples were separated on 4-12% Bis-Tris gels with the addition of reducing buffer and heating for 5 min at 95^oC.
After electrophoresis, protein bands were transferred onto nitrocellulose (NC) membranes, blocked with 5% non-fat milk protein in PBS and probed with anti-peptide mice antibodies. After washing, the NC membrane Goat anti-mouse-IRDye conjugate at 1:10k dilution was added and incubated at RT for 45min. The NC membrane was washed, and the bands were visualized using the Odyssey Infrared Imaging System (LI-COR).

5 Development of Lateral Flow Immunoassay

Ten microliters of colloidal gold conjugated anti-mouse antibody were added to an equal volume of 10% alkali-treated casein, mixed, and placed onto a conjugate pad. Diluted anti-peptide mouse primary antibody (1:10 & 1:20) and control mouse antibody in a final volume of 150 µL in PBS were placed onto the sample application pad. The immunocomplex (primary Abs and gold nano-particle conjugated 2nd anti-mouse antibody) migrated up the membrane by capillary forces into the absorbent pad and was captured by biotinylated peptide on the test (T) point. The test results were evaluated visually after 5 minutes. The limit of detection (LOD) was calculated to the highest serum dilution at 1:20 dilution.



RESULT (S)

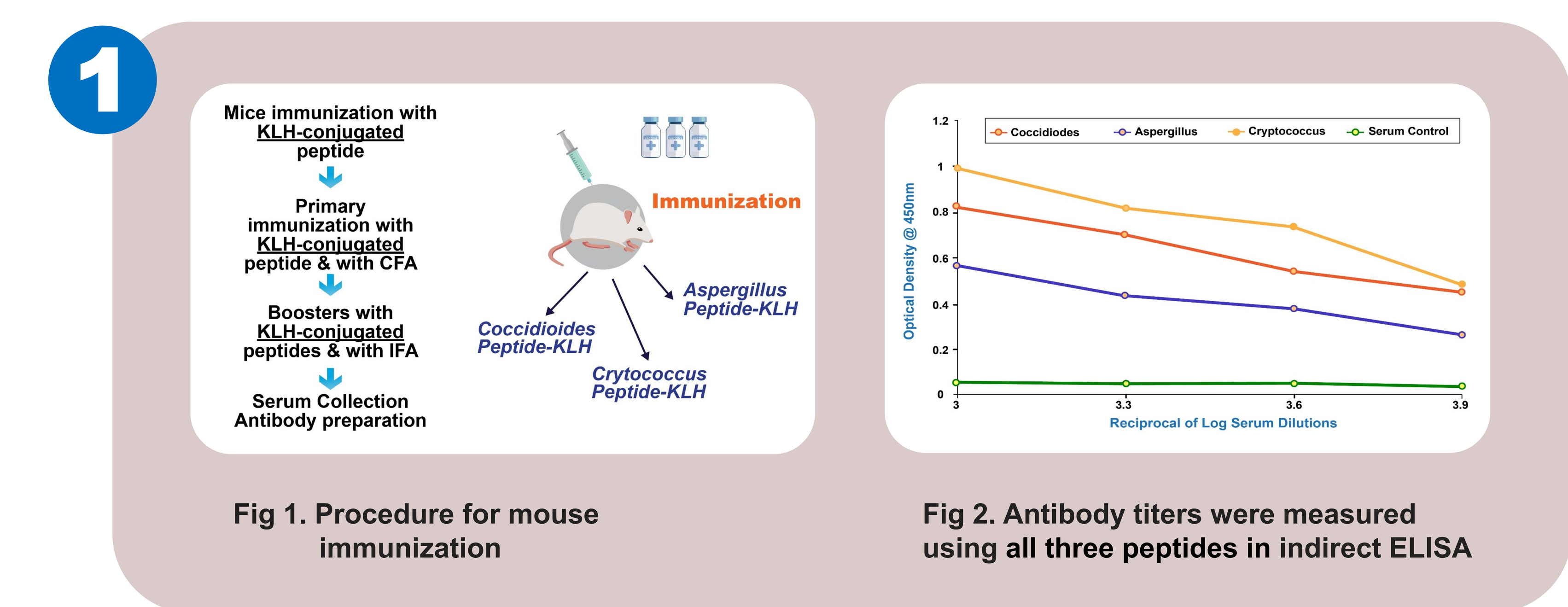


Fig 1. Procedure for mouse immunization

Fig 2. Antibody titers were measured using all three peptides in indirect ELISA

RESULT (S) Cont…

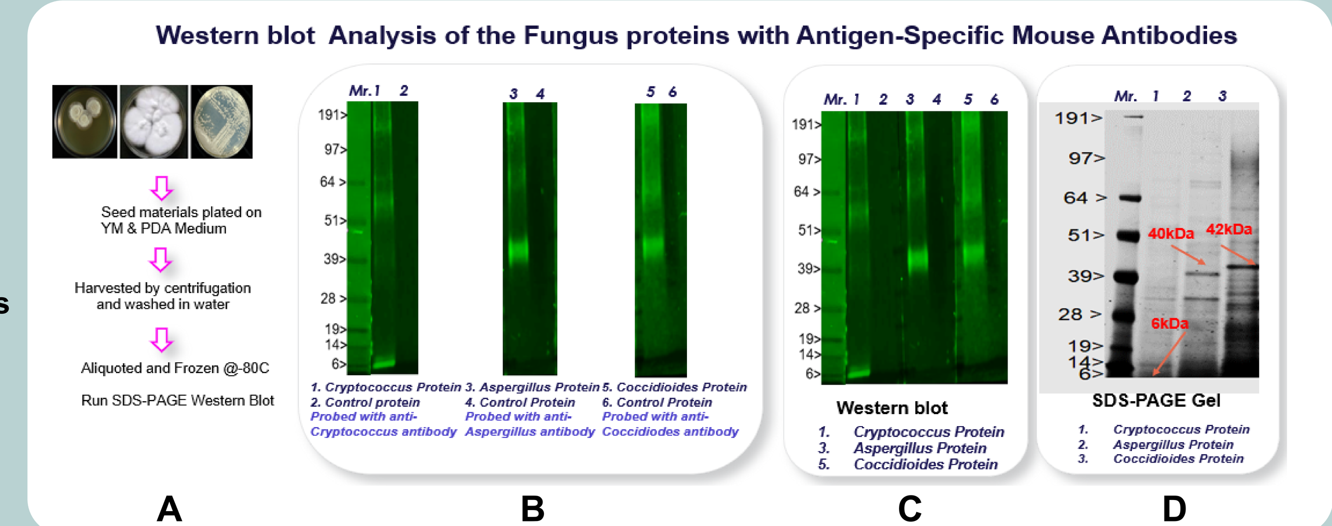


Fig 3. Fungus proteins & WB with mouse Abs

A: Fungal culture and native protein extraction for WB & SDS-PAGE analysis. **B:** WB analysis of anti-peptide mice *antibodies* on *native proteins* 1. Cryptococcus protein 2. Control protein, 3. Aspergillus protein 4. Control protein, 5. Coccidioides protein 6. Control protein. Abundant epitopes were located on 6, 40 and 42 kDa molecular weight native proteins. **C.** Western blot of NC membranes placed together **D:** SDS-PAGE analysis of Cryptococcus, Aspergillus and Coccidioides native proteins.

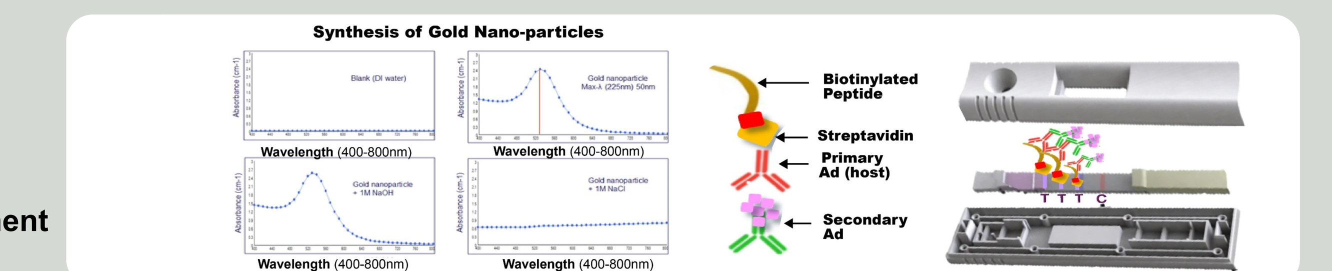


Fig 4. The LFIA development

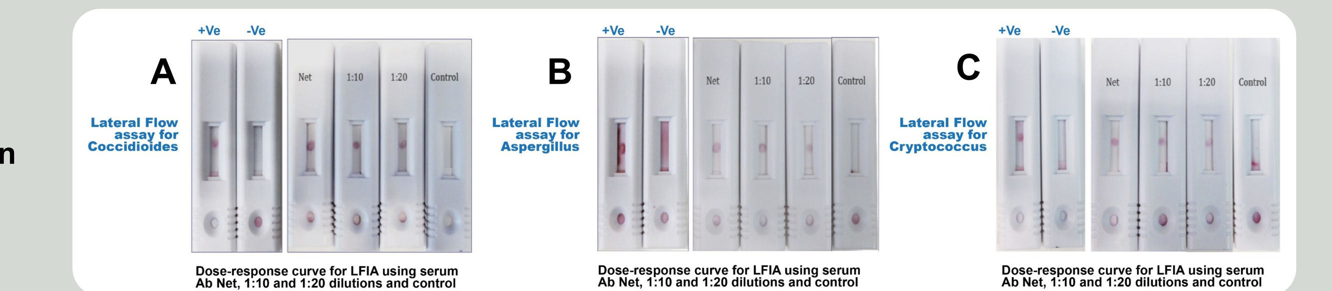


Fig 5. Assay validation

Fig 4. Synthesis of Gold nano-particles and LFIA development
Fig 5. LFIA rationally designed using biotinylated peptides directly immobilized on streptavidin coated NC membrane. The diluted anti-peptide primary antibody (1:10 and 1:20) bound to secondary antibody (anti-mouse) conjugated with colloidal gold nano-particles. The test results were visually detected in less than 5 min. The visual limit of detection (vL OD) was determined at 1: 20 serum dilution for all three anti-peptide antibodies. **A:** LFIA for Coccidioides **B:** LFIA for Aspergillus **C:** LFIA for Cryptococcus

SUMMARY

In the present study, we described a rapid, and easy-to-use multiplex LFIA for the serodiagnosis of aspergillosis, *cryptococcosis and* coccidioidomycosis, *in clinical samples.* The test can be performed within 5 min and evaluated by the naked eye. In this Proof-of-Concept (POC) study, we have shown how the synthetic immunodominant peptides coupled to the carrier protein Keyhole Limpet Hemocyanin (KLH) elicited mice antibodies against conserved epitopes from major functional area of the fungal native proteins. In addition, the LFIA test strip incorporated streptavidin immobilized biotinylated peptides as capture reagents for primary antibody and were revealed by gold nano-particles conjugated to anti-mouse secondary antibody for multi-antibody detection or multiplexing. The use of biotin/streptavidin interactions permitted more efficient detection of specific anti-peptide antibodies. The assay was initially validated with a 1: 20 serum dilution. Although the LFIA described here is at the prototype stage, we believe we have demonstrated the potential of lateral-flow technology for the detection of fungal infections. Moreover, we also believe that the LFIA could be used for the military community in the field level by untrained personnel.

ACKNOWLEDGEMENT (S)

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