Optimizing protocols and strategies for the measurement of antimalarial drug levels using ELISA-based methods

Amel O.A. Ahmed, Shamim Mohammad, Biniam Hagos, Maha Amer, Sujatha Rashid, Rebecca Bradford, and Robert E. Molestina

ATCC Federal Solutions, American Type Culture Collection, Manassas, VA 20110, USA

Email: aahmed@atcc.org

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BACKGROUND

- In malaria-endemic areas, when drug levels must be evaluated for pharmacokinetic profiling, drug efficacy trials, or pharmacovigilance purposes, samples are often sent to labs with High-Performance Liquid Chromatography (HPLC) capacity.
- HPLC, the gold standard for quantitating antimalarial drug level is unsuitable for rapid analysis of antimalarial drugs at point-of-care/point-of-purchase in resource-deprived settings where such drugs are commonly used and run a high risk of drug counterfeit.
- As an alternative to HPLC, simple, quick, inexpensive, and sensitive methods (such as ELISA) are needed to measure levels of active drugs.
- This study focuses on the antimalarial compound lumefantrine (LM), expanding on previous results obtained from a 'Proof-of-Concept' study in which we used a commercially available monoclonal antibody against chloroquine (CQ) in ELISA-based methods to detect and quantify CQ levels in different media.
- As a small molecule, LM is nonimmunogenic and will elicit an immune response only when coupled with macromolecules such as proteins to make immunogenic conjugates, a prerequisite to immunization of mice and ELISA.

Objectives

- Development of reagents and their optimization in an in-house ELISA
- Anti-LM antibody production by immunizing mice with a lumefantrine-based hapten conjugated to a carrier protein.
- Optimization of ELISA-based methods with developed reagents to detect LM.

MATERIALS & METHODS

Preparation of Protein-Hapten conjugates

- **Antimalarial compounds.** Lumefantrine (LM) and an LM-based hapten (LMH) from Sigma-Aldrich.
- **Synthesis of conjugates:**
  - Excess of the hapten (LMH) was reacted with the carrier protein (BSA) using a standard glutaraldehyde coupling procedure (Figure 1)
  - Six conjugates were synthesized at 1:5 and 1:20, 1:50, 1:100, 1:200, and 1:500 (BSA:LMH) and designated M1- M6, respectively.

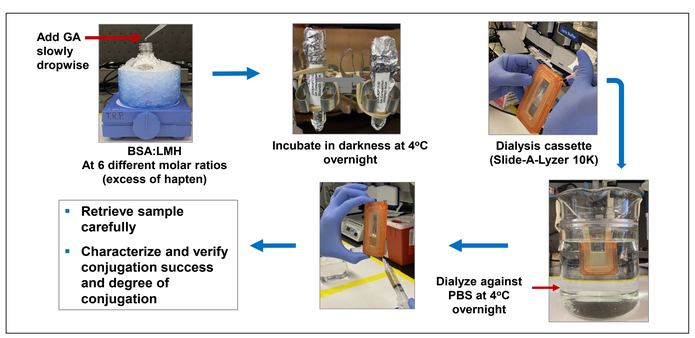

Figure 1. Glutaraldehyde Coupling Procedure

- Anti-LM antibodies were produced by immunizing balb/C mice with a lumefantrine-based hapten conjugated to a carrier protein as shown in Figure 2.

TECHNICAL APPROACH SUMMARY

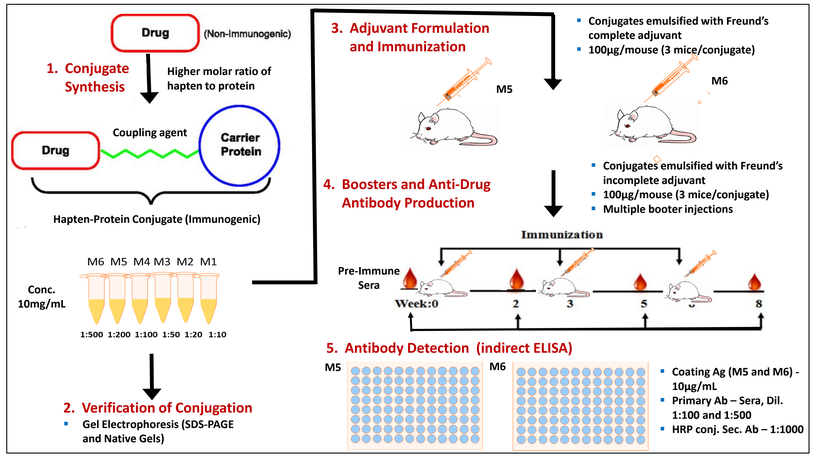

Figure 2. Production of anti-LM antibodies *by immunizing mice with a lumefantrine-based hapten conjugated to a carrier protein (BSA-LMH)*

RESULTS

Characterization and verification of Protein-Hapten Conjugates

- Protein-hapten conjugates were characterized by following physical techniques to determine conjugation success and the degree of conjugation.
- SDS-PAGE and native gel electrophoresis were used to verify conjugation (Figure 3).
- We characterized different conjugates at various molar ratios to obtain an optimum molar ratio of protein-hapten conjugate for immunization purposes.
- Conjugates M5 and M6 were selected for immunization of mice.

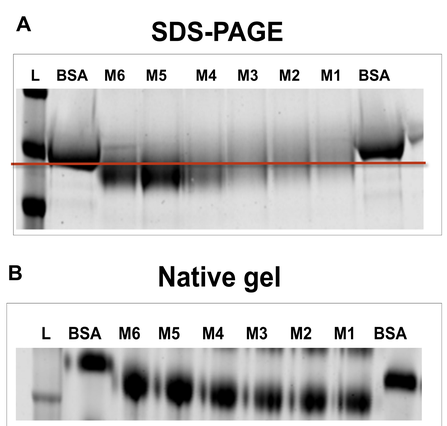

Figure 3. Gel Electrophoresis*. **Panel A. SDS-PAGE** gel shows BSA-LMH molecules with higher conjugation density (M5 and M6) have moved further on the gel, as they are more negatively charged in comparison to conjugates prepared with lower molar ratios of protein to hapten (M1-M4).*

***Panel B. Native gel** shows well-defined bands with gradual increments for each hapten with an increase in molar ratio.*

RESULTS

Measurement of titers of anti-lumefantrine antibodies in antisera

- Coated plates with conjugated haptens (BSA-LMH), hapten (LMH), and protein (BSA) (10 µg/mL)
- Incubated plates with test sera (1:500 dilution)
- Then, incubated plates with secondary antibody (1:1,000 dilution) followed by the addition of TMB substrate for color development.
- Measured optical density at 450 nm using ELISA plate reader (Molecular Devices, USA).

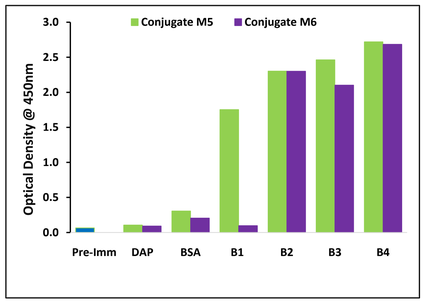

Figure 4. Titers of anti-Lumefantrine antibodies in antisera. *Mice were immunized subcutaneously with the hapten-protein conjugates M5 and M6 (100 µg each conjugate), respectively, emulsified with Freund's complete adjuvant. This was followed by the 1st booster of the same dose with incomplete Freund's adjuvant on Day 21 and then at intervals of 7 days. Mice were bled on the 7th day after each booster, and the antibody titers were determined by ELISA.*

SUMMARY

- Critical reagents (conjugates) were developed to detect LM using ELISA which is easier to access and cheaper than HPLC.
- Conjugation of hapten to protein created a LM hapten (immunogen) for immunization of mice.
- High-affinity antibodies were produced in mice against LM for use in simple ELISA for the detection of LM.
- Next phase of the study will use this ELISA-based method for LM detection and quantification in different clinical sample matrices.
- This simple assay will greatly assist public health workers to efficiently and cost-effectively undertake pharmacokinetic profiling, pharmacovigilance and monitor drug resistance and drug failure, and in the pre-screening of subjects for drug efficacy trials.

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