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Isolation and characterization of a novel anti-salbutamol chicken scFv for human doping urinalysis

Warren Lee[a], Ali Syed A.[b], Chiuan Yee Leow[a], Soo Choon Tan[a], Chiuan Herng Leow[a,*]

[a] Institute for Research in Molecular Medicine (INFORMM), Universiti Sains Malaysia (USM), 11800, Minden, Penang, Malaysia
[b] University of Louisiana at Lafayette, 104 E University Ave, Lafayette, LA 70504, United States

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ABSTRACT

Anti-salbutamol antibodies remain as important tools for the detection of salbutamol abuse in athletic doping. This study evaluated the feasibility and efficiency of the chicken (*Gallus gallus domesticus*) as an immunization host to generate anti-salbutamol scFv antibodies by phage display. A phage display antibody library was constructed from a single chicken immunized against salbutamol-KLH conjugate. After a stringent biopanning strategy, a novel scFv clone which was inhibited by free salbutamol recorded the highest affinity. This scFv was expressed as soluble and functional protein in *Escherichia coli* T7 SHuffle Express B (DE3) strain. Cross-reactivity studies of the scFv towards other relevant β2-agonists revealed that the scFv cross-reacted significantly towards clenbuterol. The determined IC_{50} of the scFv towards the two β2-agonists were; IC_{50} salbutamol = ~0.310 μg/ml, IC_{50} clenbuterol = ~0.076 μg/ml. The generated scFv demonstrated poor stability based on accelerated stability studies. The scFv was used to develop an competitive indirect ELISA (LOD = 0.125 μg/ml) for detection of parent salbutamol in spiked human urine (n = 18) with ~83.4% reliability at the cut-off of 1 μg/ml currently implemented by WADA and may be of potential use in human doping urinalysis.

Introduction

The frequent and ongoing abuse of β2-agonists as performance enhancers have resulted in numerous bans prohibiting their use in athletics as it is thought to confer an unfair performance advantage. Salbutamol is classified as one of the less potent β2-agonists however [1,2], its relative inexpensiveness renders it the drug of choice for such unsanctioned use [3]. Current detection methods aim at distinguishing orally-administered (prohibited) and inhaled salbutamol (allowed upon declaration) in asthmatic human athletes. While this has proven difficult as urinary salbutamol concentrations may overlap between the two administration routes, inhaled salbutamol generally does not exceed 1 μg/ml [4]. As such the World Anti-Doping Agency (WADA) has set a cut-off concentration of 1 μg/ml of total salbutamol (parent drug + 4-o'-sulfated metabolite) whereby concentrations exceeding this cut-off are considered an adverse analytical finding and may implicate a drug abuse scenario [5]. Consequently, a number of standardized methods for the detection of salbutamol have been established. Of these, the currently employed methods include liquid/gas chromatography coupled with mass-spectrometry (LCMS/GCMS) and immunoassays. For rapid, mass-screening purposes however, immunoassay remains as the method of choice. Immunoaffinity chromatography (IAC) has also been employed for the isolation of salbutamol followed by the detection by either LCMS or GCMS [6].

Currently available anti-salbutamol antibodies are mostly in the polyclonal format derived from sheep and rabbits [7–9]. When generating antibodies, the cross-reactivity of the generated antibody has to be accounted for and is generally perceived as a negative attribute on immunoassays; that is, an indication of poor specificity. For polyclonal antibodies, the heterogeneity of the antibody preparation usually contributes to cross-reactivity against highly similar antigens. For example, polyclonal anti-salbutamol antibodies have shown significant cross-reactivity towards clenbuterol [7–9]; which is structurally similar but more pharmacologically potent [1]. For small molecular weight targets however, the cross-reactivities of polyclonal antibodies and their monoclonal derivatives towards similar compounds do not differ significantly [10]. Thus, regardless of their clonality, the cross-reactivities of anti-β2-agonist antibodies have been efficiently exploited for simultaneous detection of multiple β2-agonists. A key advantage of monoclonal antibodies is that the laborious and prolonged maintenance of mammalian hosts to maintain high-titre polyclonal antisera is negated. Furthermore, the isolation of an antigen-specific antibody-

* Corresponding author.
E-mail addresses: lxl13_inf029@student.usm.my (W. Lee), saa3597@louisiana.edu (A. Syed A.), yee.leow@usm.my (C.Y. Leow), sctan@usm.my (S.C. Tan), herng.leow@usm.my (C.H. Leow).

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secreting clone provides and inexhaustible supply of antibodies. An example of a recently developed monoclonal anti-salbutamol antibody was by Xie et al. [11]. However, the hybridoma technology used in the study remains a slow and technical process requiring animal tissue-culture expertise.

Phage display offers a relatively inexpensive, fast and efficient method for high-throughput isolation of specific antibodies. The generation and use of mouse phage display antibody libraries to isolate antibodies against β-agonists has been reported [12,13]. However, generating phage display libraries from such conventional mammals require multiple degenerate primer pairs for efficient amplification of the V_H and V_L genes to accurately represent the entire antibody repertoire for a sufficiently diverse library. The use of degenerate primers may lead to biased PCR amplification resulting in loss of sequence diversity and potentially specific-binding clones within the generated library. The use of avian species particularly chickens (*Gallus gallus domesticus*) has circumvented this problem since amplification of the V_H and V_L genes require only a single primer pair respectively while simplifying the library construction process [14–16].

The use of chickens is an attractive alternative for the generation of monoclonal antibodies by phage display. Furthermore, only a few studies have investigated the use of chickens to isolate anti-hapten antibodies [17]. Therefore, this study reports the attempt to determine the efficacy of using chickens to generate anti-salbutamol scFv antibodies by phage display and to characterize the scFv for potential downstream immunodiagnostic applications. The scFv antibody was used to develop a competitive indirect ELISA for the detection of salbutamol in spiked human urine samples to demonstrate and evaluate its potential as an immunoanalytical tool for doping urinalysis.

Materials and methods

Raising anti-salbutamol antibodies in chicken

Hapten conjugate synthesis

Salbutamol carrier protein conjugates were synthesized using the mixed anhydride method as previously described with minor modifications [11,18]. Salbutamol hemisulphate salt (Sigma, USA) was mixed at room temperature with succinic anhydride (Sigma, USA) forming the salbutamol benzylic succinate which was then coupled to each of the following carrier proteins; bovine serum albumin (BSA), keyhole limpet hemocyanin (KLH), Ovalbumin (OVA) and bovine thyroglobulin (BTG) using the mixed anhydride (MA) method. BSA, OVA and BTG were obtained from Sigma (USA). KLH was obtained from Pierce (USA). The SAL-BSA/KLH/OVA/BTG conjugates were dialyzed against 0.01 M PBS, quantified using the Bradford protein concentration assay and stored at −20 °C until further use.

Animal immunization

All work involving the use of animals described herein was conducted based on the guidelines provided by the National Institute of Health guide for the care and use of laboratory animals [19] and approved by the Universiti Sains Malaysia (USM) Animal Ethics Committee (Ethics approval no.: USM/IACUC/2017/(110) (890)). A White leghorn chicken was immunized with the SAL-KLH conjugate as KLH is known to illicit higher immune responses. Priming immunization was performed by mixing 200 μg of SAL-KLH in 1 part saline with 3 parts of Complete Freund's Adjuvant (CFA) (Pierce, USA) in a total volume of 800 μl. Injections were administered intramuscularly into the breast muscle at four separate sites. For boosting; only 100 μg of SAL-KLH was used while CFA was substituted with Incomplete Freund's Adjuvant (IFA). A total of three boosts were performed at 1-month intervals.

Antisera specificity by ELISA

For ELISA, 4 μg/ml SAL-OVA conjugate was coated onto 96-well polystyrene microtitre plates (Corning, USA) in carbonate coating buffer followed by blocking with 1% BSA. Sera was diluted 1:20000 in 0.01 M PBS supplemented with 0.05% Tween-20 (PBST) and incubated in the coated microtitre plates. Plates were washed thrice with PBST and probed with 1:5000 goat anti-chicken IgY Fc-HRP conjugate (Invitrogen, USA). After a final wash, colorimetric development was performed by addition of 3,3,5,5-tetramethylbenzidine (TMB) substrate (Biomics, Malaysia), reaction stopped with HCl and the absorbance was measured at 450 nm using the Tecan Infinite M2000 spectrophotometer (Tecan, Austria). Antisera from each boost was also analysed for specificity and affinity towards free salbutamol by challenging the sera with 0.5 μg/ml of salbutamol in the first incubation step. After serum antibody titres and specificity were confirmed by ELISA, the animal was euthanized and the spleen was harvested for antibody library construction.

Phage display antibody library construction and scFv isolation

Construction of immunized chicken phage display library

The phage display antibody library was constructed as previously described by Andris-Widhopf et al. [14]. Approximately 2.0×10^8 spleenocytes were used for total RNA extraction with TRIzol reagent (Invitrogen, USA) and cDNA was synthesized using the Superscript III Reverse Transcription Kit (Invitrogen, USA) according to the manufacturer's instructions. Amplification of the V_L genes was performed using KAPA Taq Polymerase (KAPA Biosystems, South Africa) with PCR conditions previously described. Variable V_H genes were amplified using Q5 High Fidelity DNA Polymerase (NEB, USA) with minor modifications. A touchdown PCR was incorporated: initial denaturation; 94 °C for 5 min, 10 cycles of denaturation; 94 °C for 30s, annealing; 65 - 50 °C for 30s, elongation; 72 °C for 1 min. This was then followed by 25 cycles of denaturation; 94 °C for 30s, annealing; 56 °C for 30s, elongation; 72 °C for 1 min and a final elongation; 72 °C for 10 min. Multiple PCR reactions from the V_H and V_L amplifications were pooled separately, ethanol precipitated, electrophoresed and gel purified using the MN PCR and gel purification Kit (Macherey-Nagel, Germany). Full length scFv was assembled by SOE PCR using Vent DNA Polymerase (NEB, USA). Multiple SOE PCR reactions from the scFv assembly were pooled, ethanol precipitated, electrophoresed and gel purified as described earlier. The purified scFv was digested with *Sfi*I (Fermentas, USA), purified and ligated using T4 ligase (Invitrogen, USA) into the pComb3XSS phagemid vector. The ligated product was ethanol precipitated thrice and electroporated into electrocompetent *E. coli* TG1 (Lucigen, USA). Two electroporations were performed and pooled. Electroporated cells were plated out onto 2xYT agar plates containing 0.4% glucose and 100 μg/ml ampicillin (2× YTAG) and incubated at 25 °C for 16 h. The transformed bacterial lawn was scraped off the 2xYTAG plates, and resuspended in 2xYTAG medium supplemented with 15% glycerol and stored at −80 °C as the library glycerol stock prior to phagemid rescue. Library quality was assessed by initial library size determination, colony PCR screening for full-length scFv and diversity determination by *Bst*NI fingerprinting.

Phagemid rescue and biopanning for salbutamol-specific phage-scFv

The library glycerol stock was inoculated into 2× YTAG medium and grown at 37 °C with shaking to $O.D._{600}$ = 0.4–0.5. Phagemids were rescued by infection of the TG1 culture with KM13 helper phage at 37 °C for 30 min. The infected culture was centrifuged and the bacterial pellet was resuspended in 2xYT medium containing 50 μg/ml kanamycin and 100 μg/ml ampicillin (2× YTAK). The culture was incubated at 30 °C for 16–18 h with shaking for production of phage particles. Phages were purified from the culture supernatant by precipitation with 1/5 (v/v) of 20% PEG 6000/2.5 M NaCl. Precipitated phage was resuspended in 0.01 M PBS +15% glycerol, titrated and used directly for biopanning.

A biopanning strategy was devised in which the salbutamol carrier protein conjugates would be different for each panning round.