Journal of Microbiological Methods 116 (2015) 53–59

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

Expeditious screening of candidate proteins for microbial vaccines

Rahat Zaheer, Cassidy L. Klima, Tim A. McAllister *

Lethbridge Research Centre, Agriculture and Agri-Food Canada, Lethbridge, AB T1J 4B1 Canada

ARTICLE INFO

Article history:
Received 5 June 2015
Received in revised form 26 June 2015
Accepted 27 June 2015
Available online 3 July 2015

Keywords:
Antigen
Vaccine
ELISA
Immunoreactivity
Cell-free protein expression

ABSTRACT

Advancements in high-throughput "omics" technologies have revolutionized the way vaccine candidates are identified. Now every surface expressed protein that an organism produces can be identified *in silico* and possibly made available for the rapid development of recombinant/subunit vaccines. However, evaluating the antigenicity of a large number of candidate proteins is an immense challenge, typically requiring cloning of several hundred candidates followed by immunogenicity screening. Here we report the development of a rapid, high-throughput method for screening candidate proteins for vaccines. This method involves utilizing a coupled, cell-free transcription–translation system to screen tagged proteins that are captured at the C-termini using appropriate ligand coated wells in 96 well ELISA plates. The template DNA for the cell-free expression is generated by two sequential PCRs and includes gene coding sequences, promoter, terminator, other necessary cis-acting elements and appropriate tag sequences. The process generates expressible candidate proteins containing two different peptide tags at the N- and the C-termini of the protein molecules. Proteins are screened in parallel for their quantity and immunoreactivity with N-terminal tag antibodies and antisera raised against the pathogen of interest, respectively. Normalization against the total detectable bound protein in the control wells allows for the identification of highly immunoreactive candidates. For this study we selected 30 representatives of >300 potential candidate proteins from *Mannheimia haemolytica*, a bacterial agent of pneumonia in feedlot cattle for expression with N-terminal Strep-II and C-terminal His(x6)-tag and evaluated their relative immunoreactivities using Strep-tactin-HRP and rabbit antisera generated against *M. haemolytica*. Using this system we were able to swiftly and quantitatively analyze and rank the suitability of proteins to identify potentially viable vaccine candidates, with the majority of the high ranking candidates being associated with virulence and pathogenicity. The system is adaptable to any bacterial target and presents an alternative to conventional laborious cloning, expression and screening procedures.

1. Introduction

Bacterial infections continue to be a major cause of disease in both humans and livestock. Although immunization is the most effective and efficient method of preventing microbial diseases, only a fraction are managed by vaccines. As interpreted by Rappuoli (2007), the majority of vaccines licensed to date have been developed based on Pasteur's principles of "isolate, inactivate and inject" the causative disease agent. With the advent and advancement of genome sequencing, vaccine research has embraced a more innovative genome-based dimension called 'reverse vaccinology' which promises discovering novel and unconventional antigens in addition to unraveling previously unidentified virulence agents as vaccine targets for several pathogens. During the past several years, the possibilities of selecting targets using computational approaches with integrated 'omics' data such as genomics, proteomics and metabolomics have been continuously on the rise. Among these, two *in silico* methods, comparative genomics and subtractive genomics are now being commonly used for the prediction and identification of putative drug targets in numerous pathogenic bacteria and fungi (Abadio et al., 2011; Amineni et al., 2010; Perumal et al., 2007). These advancements along with high-throughput computational methods have entirely revolutionized the way vaccine candidates are identified. Now, with reverse vaccinology a complete potential antigenic repertoire of an organism can be deduced from its genome *in silico* including surface expressed and secreted proteins with the possibility of them being generated for the rapid development of recombinant/subunit vaccines. However, evaluating the antigenicity of a large number of candidate proteins is still an immense challenge, typically requiring cloning and expression of several hundred protein candidates that require purification for immunoscreening. Conventional cell based methods of protein expression are laborious, time consuming and can still fail because of issues including low solubility due to protein aggregation, formation of inclusion bodies, toxicity to the host and instability (Rosenblum and Cooperman, 2014). In addition, efficient screening processes are required to identify a manageable number of highly antigenic candidates to be advanced into live animal trials. Here we report the development

* Corresponding author at: Agriculture and Agri-Food Canada, Lethbridge Research Center, 5403-1st Ave. South, Lethbridge, Alberta T1J 4B1, Canada.
E-mail address: tim.mcallister@agr.gc.ca (T.A. McAllister).

http://dx.doi.org/10.1016/j.mimet.2015.06.018
0167-7012/

of a rapid, high-throughput method for expressing and screening candidate proteins for vaccines. For this study protein candidates including outer membrane proteins, lipoproteins and periplasmic proteins as inferred through *in silico*-based methods from a previously sequenced genome of *M. haemolytica* were selected for screening of their immunoreactivity potential. This is a proof-of-principle study describing the development of a high-throughput *in vitro* immunoscreening assay for the selection of potent antigens and discusses the candidacy potential of identified high ranking candidates in perspective of their involvement in bacterial virulence and pathogenicity, characteristics deemed suitable for the development of vaccines against pathogens.

2. Materials & methods

2.1. Isolation of M. haemolytica genomic DNA

Genomic DNA was isolated from *M. haemolytica* strain PHL213 (NCBI genome Accession # NZ_AASA00000000) culture grown overnight from a fresh single colony in 5 mL trypticase soya broth. Briefly, cells were harvested from the culture by centrifugation at 6000 ×*g* for 5 min and the pellet was suspended in 1 mL of sterile 0.85% NaCl and centrifuged again for 1 min to wash away growth media. The washed cell pellet was resuspended in 700 μL of $T_{10}E_{25}$ (10 mM Tris-HCl pH 7.5; 25 mM EDTA). To the suspension, 175 μL of 5 M NaCl, 35 μL of 10 mg/mL Proteinase K and 44 μL of 20% SDS were added and the mixture was incubated at 65 °C, for 1–2 h until complete cell lysis occurred. The lysed mixture was extracted once with phenol, once with phenol:chloroform:isoamylalcohol (25:24:1) and twice with chloroform. Ammonium acetate (10 M) was added to the final aqueous fraction to achieve a final concentration of 0.5 M followed by the addition of 1 volume of isopropanol to precipitate the DNA. The DNA was gently spooled out, added to a new tube containing ice-chilled 70% ethanol and centrifuged at 10,000 ×*g* for 10 min to obtain a DNA pellet. The supernatant was decanted and the tube was left open to air-dry. The pellet was suspended in 100 μL of nuclease free deionized H_2O.

2.2. Cell-free protein synthesis

For the proof of principle study, thirty proteins (as described by their locus tags in Table 1) were selected representing outer membrane proteins, lipoproteins, periplasmic proteins and hypothetical proteins containing N-terminal transmembrane signal peptides as identified by SignalP and PSORTb (Petersen et al., 2011; Yu et al., 2010). The selected protein candidates included two known immunogenic outer membrane proteins, PlpE (MHA_1514) and OmpA (MHA_1054) previously identified as potential vaccine candidates against *M. haemolytica* (Confer and Ayalew, 2013; Ayalew et al., 2004) and 28 other proteins of known, predicted and unknown functions. The EasyXpress Linear Template Kit (Qiagen, Toronto, ON) was used to generate translationally active PCR products suitable for cell-free biosynthesis of protein using an *Escherichia coli*-based coupled *in vitro* transcription-translation (IVTT) system from Qiagen (EasyXpress Protein Synthesis kit). Briefly, a two-step sequential PCR procedure was employed to generate translationally active PCR products (Fig. 1) in a 96-well plate format. For the first PCR, specific primers for the protein of interest were optimally designed (Table 1) and 5′-tails appropriate for the incorporation of 5′ strep-II tag and 3′ His(x6) tag in the second PCR step, as defined by the manufacturer, were added. Genomic DNA isolated from *M. haemolytica* strain PHL213 was used as template to generate 30 individual PCR products corresponding to 30 selected genes. To increase the solubility of target proteins, the specific sequences in the primers were selected to exclude any predicted N-terminal transmembrane and periplasmic signal coding sequences from otherwise full-length genes. However, for one of the target genes *ssa1*, coding for serotype-1 specific antigen, the gene specific primers were designed to generate a truncated version (nt 83–874) of the protein as the full-length gene was very long (2.8 kb) and was unlikely to be efficiently transcribed and translated in a cell-free system. The amplifiable coding sequence lengths for selected genes varied between 240–1398 nucleotides (Table 1). The second round of PCR used amplified products from the first PCR as template according to manufacturer's instructions. The 5′-tail sequences from the primers for the 1st round of PCR now incorporated in the template acted as hybridization sites for the adapter primers used in the second PCR to generate translationally active templates consisting of T7 promoter, ribosomal binding site, gene coding sequences fused in frame with 5′ strep-II and 3′ His(x6) tags, and T7 terminator. Products from each of the two PCR steps were analyzed on a 0.8% agarose gel for evaluating their size and quality.

The final PCR products were added to the IVTT reactions (Qiagen EasyXpress Protein Synthesis kit) without any further purification, according to the manufacturer's instructions with slight modifications. Briefly, a 25 μL reaction volume was set up for each target protein expression using 0.4–0.5 μg of the final PCR product as expression template and the reaction plate was incubated in a thermo-mixer at 33 °C for 90 min with gentle shaking at 300 rpm. To validate the expressed products through Western blotting for the presence of-His(x6) and Strep-II tags, and for the detection of immunoreactivity against *M. haemolytica* antisera, a 9 μL aliquot (3 μL for each blot) was taken and stored in SDS-polyacrylamide sample buffer at −20 °C for later use. The EasyXpress Positive-Control DNA in combination with its primers all supplied in the Qiagen's EasyXpress Linear Template kit were used for the PCR and *in vitro* protein expression steps as a positive control. The resulting protein, a 32 kDa elongation factor EF-Ts with a C-terminal His(x6) tag was used as a negative control for the downstream steps involving immunoreactivity screening ELISAs.

2.3. Quantitative immunoreactivity screening

Proteins were screened with ELISA, in parallel for their relative quantity and immunoreactivity with Strep-tactin-HRP and polyclonal antisera previously raised in rabbits against formalin-inactivated whole cells of *M. haemolytica*, serotype 1 (Klima et al., 2011) with the assays performed in triplicate. Pre-immune rabbit serum did not show cross reactivity with *M. haemolytica* proteins. For setting up the immunoreactivity ELISAs, briefly, 185 μL of solubilization buffer (50 mM sodium phosphate [pH 8.0], 300 mM NaCl, 10 mM imidazole and 1.2% Triton X-100) were added to the 25 μL completed IVETT reaction in each well and thoroughly mixed by gently pipetting up and down, without introducing air bubbles with a multi-channel pipette. One hundred microliters of solubilized protein mixture was added to each of the two Ni^{+2} coated plates (Pierce, Cat # 15142 or 15442) and incubated for 1 h at room temperature with shaking at 400 rpm in a plate thermomixer. The mixture containing the unbound proteins was removed and wells were washed 3 times for 5 min each, with 200 μL of wash buffer (50 mM sodium phosphate [pH 8.0], 300 mM NaCl, 30 mM imidazole). Strep-tactin-HRP conjugate (product # 161-0382; Bio-Rad Laboratories, Inc. Hercules, CA, USA) was diluted 1:20,000 with antibody dilution buffer (Phosphate buffered saline (PBS) containing 0.05% Tween-20 and 2% bovine serum albumin) and added (150 μL) to the wells in one of the plates containing Ni^{+2} bound proteins (referred to as Strep–ELISA plate; 'Control' plate in Fig. 1). The plate was incubated for 1 h at room temperature with shaking at 400 rpm. To the Ni^{+2} bound proteins in the second plate (denoted as MH–ELISA plate; 'Test' plate in Fig. 1) polyclonal antibodies raised in rabbits against *M. haemolytica* were added at 1:2000 dilution in antibody dilution buffer and incubated for 1 h at room temperature with shaking at 400 rpm. Following incubation, both plates were washed 3 times for 5 min each using 200 μL of ELISA-wash buffer (PBS containing 0.05% Tween-20). The Strep–ELISA plate was processed for detection using SIGMAFAST™ OPD tablets (P9187, Sigma-Aldrich, Oakville, ON, Canada) according to the manufacturer's instructions. To the MH–ELISA plate, 200 μL of goat anti-rabbit IgG-HRP antibody diluted 1:10,000 in antibody dilution buffer were added and incubated at room temperature for 1 h with shaking