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Research paper

In silico identification and high throughput screening of antigenic proteins as candidates for a *Mannheimia haemolytica* vaccine

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ABSTRACT

This study examined the use of comparative genomic analysis for vaccine design against *Mannheimia haemolytica*, a respiratory pathogen of ruminants. A total of 2,341genes were identified in at least half of the 23 genomes. Of these, a total of 240 were identified to code for N-terminal signal peptides with diverse sub-cellular localizations (78 periplasmic, 52 outer membrane, 15 extracellular, 13 cytoplasmic membrane and 82 unknown) and were examined in an ELISA assay using a coupled-cell free transcription/translation system for protein expressionwith antisera from cattle challenged with serovars 1, 2 or 6 of *M. haemolytica*. In total, 186 proteins were immunoreactive to at least one sera type and of these, 105 were immunoreactive to all sera screened. The top ten antigens based on immunoreactivity were serine protease Ssa-1 (AC570_10970), an ABC dipeptid transporter substrate-binding protein (AC570_04010), a ribonucleotide reductase (AC570_10780), competence protein ComE (AC570_11510), a filamentous hemagglutinin (AC570_01600), a molybdenum ABC transporter solute-binding protein (AC570_10275), a conserved hypothetical protein (AC570_07570), a porin protein (AC569_05045), an outer membrane assembly protein YeaT (AC570_03060), and an ABC transporter maltose binding protein MalE (AC570_00140). The framework generated from this research can be further applied towards rapid vaccine design against other pathogens involved in complex respiratory infections in cattle.

1. Introduction

Vaccination is one of the most efficient and cost-effective management strategies to control and eliminate disease (Rappuoli and Aderem, 2011). With significant advancements in “omics” technologies over the past two decades, approaches used for vaccine design have also evolved. Traditional methods used to generate first and second generation vaccines have focused on the whole organism based on the principals of “isolate, inactivate and inject” proposed by Pasteur in the 19th century (Doolan et al., 2014). The availability of whole genome sequencing has shifted the focus towards *in silico* antigen prediction for the development of third generation vaccines. This reverse vaccinology (RV) approach starts from the genome rather than the whole organism and identifies the entire catalog of proteins that an organism has the potential to express at any point in time. This strategy has the added benefit of being applicable to cultivatable and non-cultivatable species (Rappuoli, 2001).

In silico prediction of vaccine candidates relies on software that can screen whole genome sequences and identify coding DNA. The sub-cellular localization of these sequences is determined by bioinformatic tools that identify signatures like signal peptides, transmembrane helices or cell-wall anchor motifs associated with surface exposed or secreted proteins (Bertholet et al., 2014). These proteins are most likely to be accessible to the immune system and thus make good vaccine targets. Comparative genomics and pan-genome analysis can be coupled with this strategy to ensure the antigens selected provide maximum coverage for a universal vaccine against a diverse species or to avoid cross reactivity against non-target commensal species (Moriel et al., 2008).

The strategy to start from the genome rather than the organism was first proposed by Rappuoli (2000). Since the conception of RV, progress in genomic, proteome, and transcriptome analysis have had a massive impact on the speed that novel antigens are being identified (Rappuoli and Aderem, 2011). However, challenges still exist with evaluating the antigenicity of a large number of vaccine candidates. Traditional antigen production strategies require the cloning and expression of several

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Table 1
Strains of *Mannheimia haemolytica* used in pan-genome analysis.

Strain	Accession Number	Serovar	Isolate origin
M. haemolytica L02A	LFXX00000000.1	1	Texas
M. haemolytica L044A	LFXY00000000.1	1	Nebraska
M. haemolytica 157-4-1	LFYD00000000.1	1	Alberta
M. haemolytica 535A	LFYB00000000.1	1	Alberta
M. haemolytica T2	LFXW00000000.1	2	France
M. haemolytica L033A	LFXZ00000000.1	2	Nebraska
M. haemolytica 587A	LFYC00000000.1	2	Alberta
M. haemolytica L038A	LFYA00000000.1	6	Alberta
M. haemolytica T14	LFXV00000000.1	6	France
M. haemolytica H23	AOGP00000000.1	6	Alberta
M. haemolytica 3927A	LFYE00000000.1	6	Alberta
M. haemolytica USDA-ARS-USMARC 2286	NC_021883.1	n/a	Nebraska
M. haemolytica D174	NC_021739.1	6	Iowa
M. haemolytica D171	NC_021738.1	2	Iowa
M. haemolytica D153	NC_021743.1	1	Iowa
M. haemolytica MhSwine2000	ATTA00000000.1	1	Iowa
M. haemolytica MhBrain2012	ATSZ00000000.1	1	Georgia
M. haemolytica D38	AUNL00000000.1	6	Iowa
M. haemolytica D35	AUNK00000000.1	2	Iowa
M. haemolytica D193	ATSY00000000.1	1	Iowa
M. haemolytica M42548	NC_021082.1	1	Pennsylvania
M. haemolytica USDA-ARS-USMARC-183	NC_020833.1	1	Kansas
M. haemolytica USDA-ARS-USMARC-185	NC_020834.1	6	Kansas
M. haemolytica PHL213	NZ_AASA00000000.1	1	n/a
M. haemolytica Bovine A2	NZ_ACZY00000000.1	2	n/a
M. haemolytica Ovine A2	NZ_ACZX00000000.1	2	n/a

hundred proteins (Pizza et al., 2000) and their purification prior to evaluation in immunogenicity assays. These methods are often laborious and challenging due to the many complications associated with expression of outer membrane proteins including issues of low solubility, the formation of inclusion bodies, protein instabilities and toxicity to the expression host (Rosenblum and Cooperman, 2014). As an alternative to these conventional approaches, we have previously developed a rapid, high-throughput method for expressing and screening vaccine candidate proteins and have demonstrated its efficiency in selection of potent antigens in a proof-of-principle study (Zaheer et al., 2015). Using this method, the present study employed a RV approach to identify conserved antigens in pathogenic strains of *M. haemolytica* for the purpose of developing a bovine vaccine against this organism.

2. Material and methods

2.1. Antigen candidate selection

Pan-genome analysis was used to identify groups of coding genes present in the whole genome sequence of 23 *M. haemolytica* isolates (Table 1). The analysis, performed used a pan-genome analysis pipeline (PGAP) (Zhao et al., 2012) and produced a gene cluster file that grouped all orthologues present in the dataset based on a cut-off value of 95% sequence identity over 90% of the sequence length. From this file, orthologous clusters were selected based on the gene occurrence in at least 50% of genomes. A single representative gene from each cluster was selected, prioritizing the *M. haemolytica* L044A genome. The *M. haemolytica* L044A genome was given priority as it had the largest genomic content among the sequenced genomes, anticipating its use as a template for amplifying the majority of target genes for downstream expression and immunoreactivity studies. If the representative gene was absent in *M. haemolytica* L044A, it was selected from the genomes of strains from serotypes 1 or 6 including *M. haemolytica* L02A, L038A or H23. The resulting group of genes was screened using SignalP (Petersen et al., 2011) to identify N-terminal signal peptides, indicative of gene products that are secreted from the cell. The TMHMM server v 2.0 was also used to identify proteins containing transmembrane helices that are typically associated with the cell membrane (http://www.cbs.dtu.dk/services/TMHMM-2.0/). The program PSORTb 3.0 (Yu et al., 2010) was used to predict the localization of each gene product within the cell. Candidates were selected based on having N-terminal signal peptides, fewer than 4 transmembrane helices, known or predicted localization in the periplasm, cell membrane, or extracellular secretion. In addition, identified lipoproteins and predicted hypothetical proteins with these desired traits were further examined in an immunoreactivity assay.

2.2. Protein expression

The EasyXpress Protein Synthesis kit (RiNA GmbH, Germany) was used to express genes encoding for protein candidates *in vitro* according to manufacture's specifications. Template for the PCR reactions used to generate selected genes originated from overnight cultures of *M. haemolytica* L044A, *M. haemolytica* L02A, *M. haemolytica* L038A or *M. haemolytica* H23 grown on tryptic soy agar (TSA) blood at 37 °C. The genomic DNA was isolated as described earlier (Zaheer et al., 2015).

According to the EasyExpress Liner Template kit instructions, a two-stage PCR process was used to generate a transcriptionally active PCR product. This product contained the coding sequence of the targeted antigen along with the regulatory elements required for optimal transcription and translation in a cell-free expression system and an N-terminal *Strep*-tag, and a C-terminal 6xHis tag. The first PCR used primers generated specifically for each coding sequence with an additional 5′ adaptor consisting of 30 nucleotides for the forward primer and 20 nucleotides for the reverse primer. These 5′ adaptors were targets for the second round of PCR that was used to add the regulatory elements and end terminal tags to the final PCR product. For the first round of PCR a two-step program was used. After an activation step of 95 °C for 30 s, 5 cycles of 94 °C for 1 min, 55° for 1 min and 72 °C for 2 min were performed followed by 35 cycles of 94 °C for 1 min, 62 °C for 1 min and 72 °C for 2 min, followed by a final 10 min extension at 72 °C. A list of the gene targets, their characteristics, and the primers used is presented in supplementary TableS1. The second round of PCR used amplicon products from the first PCR as template according to manufacturer's specifications. A two-step program was also used for this second round of PCR. After an activation step of 95 °C for 30 s, 5 cycles of 94 °C for 1 min, 50° for 1 min and 72 °C for 2 min were performed followed by 35 cycles of 94 °C for 1 min, 53 °C for 1 min and 72 °C for 2 min, followed by a final 10 min extension at 72 °C. The HotStar HiFidelity Polymerase Kit (Qiagen Canada Inc., Toronto, ON) was used for both rounds of PCR reactions.

The EasyXpress protein synthesis kit was used according to manufacturer's specifications to express the tagged proteins used for immunoreactive screening. A 40 μL reaction volume was set up for each targeted protein expression using 5.6 μL of the final product from the final PCR reaction as expression template. The expression reactions were incubated in a thermo-mixer at 33 °C for 90 min at 300 rpm.

2.3. Generation of sera against M. haemolytica for antigen screening

To generate the sera for screening antigenic candidates, three groups of three calves each were challenged twice, on day 1 and 28, with intranasal challenge of either serotype 1, 2 or 6 strains of *M. haemolytica*. The serotype 1 group inoculum consisted of a mixture of *M. haemolytica* L02A, *M. haemolytica* L044A, *M. haemolytica* 535A and *M. haemolytica* 157-4-1. The serotype 2 group inoculum consisted of a mixture of *M. haemolytica* 587A, *M. haemolytica* L033A, and *M. haemolytica* T2. And the serotype 6 group inoculum consisted of a mixture of *M. haemolytica* L038A, *M. haemolytica* H23, *M. haemolytica* T14, and *M. haemolytica* 3927A.

To prepare bacteria for intranasal challenge, 350 mL aliquots of brain heart infusion (BHI) broth were each inoculated at a 1:100