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A reverse vaccinology approach to swine dysentery vaccine development

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ABSTRACT

Swine dysentery (SD) is a mucohaemorrhagic colitis of pigs resulting from infection of the large intestine with the anaerobic intestinal spirochaete *Brachyspira hyodysenteriae*. Whole-cell bacterin vaccines are available to help control SD, but their performance has been inconsistent. This study aimed to use a reverse vaccinology approach to identify *B. hyodysenteriae* proteins for use as recombinant vaccine components. Nineteen open reading frames (ORFs) predicted to encode potential vaccine candidate molecules were identified from *in silico* analysis of partial genomic sequence data. The distribution of these ORFs among strains of *B. hyodysenteriae* was investigated by PCR, and widely distributed ORFs were cloned. The products were screened with a panel of immune pig sera, and from these a subset of conserved, immunogenic proteins was selected. Mice immunized intramuscularly with these recombinant proteins developed specific systemic antibody responses to them, and their sera agglutinated *B. hyodysenteriae* cells *in vitro*. In a pilot experiment, eight pigs were vaccinated twice intramuscularly with a combination of four of the proteins. The pigs developed antibodies to the proteins, and following experimental challenge only one developed SD compared to five of nine non-vaccinated control pigs. Although these differences in incidence were not significant, they indicated a trend towards protection using the recombinant proteins as immunogens. This study demonstrates that the reverse vaccinology approach has considerable potential for use in developing novel recombinant vaccines for SD.

1. Introduction

The anaerobic intestinal spirochaete *Brachyspira hyodysenteriae* is an important enteric pathogen. Infection of growing and fattening pigs results in swine dysentery (SD), a severe mucohaemorrhagic colitis that causes significant disruption to production (Hampson et al., 2006). Although the incidence and severity of SD can be reduced in infected herds by using antimicrobials, strains of *B. hyodysenteriae* that are resistant to various key antimicrobial agents are increasingly being encountered (Duinhof et al., 2008). The availability of an effective vaccine for SD would be an important alternative or supplement to the use of antimicrobials for control of the disease.

To date vaccines for SD have largely been based on inactivated whole cells of *B. hyodysenteriae*, but these often fail to confer a satisfactory degree of protection against infection, and do not provide adequate cross-protective immunity against strains of different serogroups (Hampson et al., 2006). Efforts have been made to identify *B. hyodysenteriae* proteins for use in subunit vaccines, but vaccination with a recombinant 38 kDa flagellar protein failed to prevent colonization in experimentally infected pigs (Gabe et al., 1995). On the other hand, vaccination with a recombinant 29.7 kDa outer membrane lipoprotein (Bhlp29.7) resulted in partial protection, with fewer animals developing disease than occurred in the control groups (La et al., 2004). This result provided evidence for the potential usefulness of recombinant proteins as

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subunits for SD vaccines, and encouraged the current investigation.

"Reverse vaccinology" uses a combination of bioinformatics analysis of whole genomic sequence data and laboratory screening to identify novel vaccine candidates for bacterial pathogens (Rappuoli, 2001). This approach was first successfully applied to *Neisseria meningitidis* serogroup B, for which conventional strategies have failed to provide an efficacious vaccine (Pizza et al., 2000).

The aim of the present study was to investigate the use of the reverse vaccinology approach to select potential vaccine candidates for *B. hyodysenteriae*, and to test a small number of these for efficacy in pigs.

2. Materials and methods

2.1. Spirochaete strains and growth

A total of 27 well-characterized *B. hyodysenteriae* strains representing the major serogroups were obtained from the collection held at the Australian Reference Centre for Intestinal Spirochaetes (ARCIS), School of Veterinary and Biomedical Sciences, Murdoch University. These comprised 19 strains from Australia, 4 from the USA, 3 from Canada, and 1 from The Netherlands. The strains were thawed and grown at 37 °C in Kunkle's pre-reduced anaerobic broth containing 2% (v/v) foetal bovine serum and 1% (v/v) ethanolic cholesterol solution (Kunkle et al., 1986).

2.2. Permissions

The experimental work with animals was conducted with the approval of the Murdoch University Animals Ethics Committee. All procedures were carried out under Australian National Health and Medical Research Council guidelines.

2.3. Genomic sequencing and in silico analysis

The genome of Australian *B. hyodysenteriae* strain WA1 (ATCC 49526) was sequenced using a shotgun sequencing approach at the Australian Genome Research Facility, University of Queensland, Australia, with an approximate six times coverage. The program Phred was used for fragment assembly (Ewing et al., 1998), with subsequent viewing using Consed (Gordon et al., 1998). Identification of open reading frames (ORFs) was carried out using Glimmer and GeneMark (Lukashin and Borodovsky, 1998; Salzberg et al., 1999). The identified ORFs were given a temporary designation starting with ORF-, with the encoded protein identified with the same number but starting with P-. For selected ORFs, searches for functional assignments were conducted using BlastP (http://ftp.ncbi.nlm.nih.gov/) performed with the non-redundant protein database (Altschul et al., 1997). Protein domains were assigned by searching against Pfam (Bateman et al., 2002) and the Conserved Domain Database (CDD) (Wheeler et al., 2001). The cut-off *E*-value was set to 10^{-7} as a default, and proteins with lower *E*-values were considered genuine homologies. Cellular localization predictions for each ORF were carried out as follows: prediction of the presence and location of signal peptides in the N-terminal 70 amino acids of an ORF, using SignalP (Version 3.0, http://www.Cbs.dtu.dk/services/SignalP/) (Bendtsen et al., 2004); prediction of protein localization sites in Gram-negative bacteria using PSORTb (Version 2.0, http://www.psort.org/psortb/) (Nakai, 2000; Gardy et al., 2005); identification of lipoprotein signatures by the Lipop program of the PSORT package (Nakai, 2000), and SpLip (Setubal et al., 2006) for specifically predicting spirochaetal lipoproteins (obtained from Setubal and Haake, Virginia Bioinformatics Institute, USA); and recognition of membrane-spanning regions, using TMpred (http://www.ch.embnet.org/software/TMPRED_form.html) (Hofmann et al., 1999). Theoretical molecular weights and isoelectric points were calculated using the Pepstats program (Emboss).

2.4. PCR amplification and sequencing

Pairs of primers that annealed to internal regions of each of 19 selected coding sequences were designed (supplementary table S1), and were used for PCR amplification from the *B. hyodysenteriae* strains. If a gene could be amplified from more than 90% of the strains examined, it was selected for cloning.

DNA sequencing was conducted using a pair of flanking primers (Supplementary table 2) that annealed to regions external to the coding sequences of the putative genes. For ORF-N17, which was greater than 1600 base pairs (bp), internal primers were used to cover the missing regions. For sequencing, whole ORFs were amplified from the genomes of 6–7 *B. hyodysenteriae* strains. The PCR products were purified using the UltraClean up Kit (Mo Bio Laboratories, Solana Beach, CA, USA), according to the manufacturer's instructions. Sequencing of the PCR products was performed using the ABI PRISM™ Dye Terminator cycles Sequencing Ready Reaction Mix (PE Applied Biosystems, Foster City, CA, USA). Sequence results were edited and compiled using SeqEd v1.0.3 (PE Applied Biosystems), then translated into amino acid sequence using the BioEdit Sequence Alignment Editor (North Carolina State University). The nucleotide and translated protein sequences for all the genes were compared to produce a pair-wise identity matrix using Clustal W (Thompson et al., 1994).

2.5. Cloning and expression of recombinant protein

ORFs were amplified from *B. hyodysenteriae* strain WA1 using primers encoding restriction endonuclease recognition sites for cloning into the *E. coli* expression vector pTrcHisA (Invitrogen, Carlsbad, CA, USA). If an N-terminal signal peptide for secretion was predicted, the corresponding nucleotide sequence was excluded. Among the selected coding sequences, ORF-H17, encoding a large protein with a predicted size of 111.05 kDa was amplified and cloned in two smaller sub-fragments (ORF-H17 N-terminus and ORF-H17 C-terminus) to facilitate subsequent expression and purification. Constructs were confirmed by sequencing using vector (pTrcHisA)-specific primer (FP: 5′-GAGGTA-