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Oral immunization of broiler chickens against necrotic enteritis with an attenuated *Salmonella* vaccine vector expressing *Clostridium perfringens* antigens

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ABSTRACT

Necrotic enteritis (NE) in broiler chickens is caused by *Clostridium perfringens* but currently no effective vaccine is available. Our previous study showed that certain *C. perfringens* secreted proteins when administered intramuscularly protected chickens against experimental infection. In the current study, genes encoding three *C. perfringens* proteins: fructose-biphosphate-aldolase (FBA), pyruvate:ferredoxin-oxidoreductase (PFOR) and hypothetical protein (HP), were cloned into an avirulent *Salmonella enterica* sv. *typhimurium* vaccine vector. Broiler chickens immunized orally with recombinant *Salmonella* expressing FBA or HP proteins were significantly protected against NE challenge. Immunized birds developed serum and mucosal antibodies to both clostridial and *Salmonella* antigens. This study showed the oral immunizing ability of two *C. perfringens* antigens against NE in broiler chickens through an attenuated *Salmonella* vaccine vector.

1. Introduction

Clostridium perfringens is the causative agent of necrotic enteritis (NE) in broiler chickens. The disease occurs when these bacteria produce large amounts of necrotizing extracellular toxins that damage the small intestine of chickens, but there is no detailed understanding of the pathogenesis of the infection. Emerging evidence is that there are NE strains distinct from non-NE strains present in the normal intestinal microflora of chickens that displace the latter in producing disease [1]. Although alpha-toxin has been shown to be important in the pathogenesis of and immunity to *C. perfringens*-associated gas gangrene [2–6], and apparently also in NE [7–11], toxin(s) other than alpha-toxin also seem to be important in the pathogenesis of NE [12]. A toxin with partial homology to beta-toxin, NetB, has recently been shown to have an apparently major role in NE pathogenesis [13]. NE is commonly prevented by the use of antimicrobials in feed or water but such a usage has in recent years raised concern for the public health because of antibiotic resistance, such that there is a ban on prophylactic use of antimicrobials in poultry production in Europe. Broiler chickens reared under intensive conditions in the absence of antimicrobial prophylaxis are at high risk to NE [14].

Although immunization offers an attractive alternative approach to antimicrobials in the control of NE, there is currently no effective vaccine available for broiler chickens and the basis of immunity to NE is not well understood. Our earlier studies showed that certain secreted proteins of virulent *C. perfringens* [alpha-toxin, glyceraldehyde 3-phosphate dehydrogenase, truncated pyruvate:ferredoxin oxidoreductase (tPFOR), fructose 1,6-biphosphate aldolase (FBA), and a hypothetical protein (HP)]-induced significant protection against challenge with virulent *C. perfringens* in birds immunized intramuscularly with each of these proteins [10]. A role for alpha-toxin in NE immunity was further supported by a recent study that demonstrated the protective property of the carboxy-terminal domain of alpha-toxin when delivered orally through an attenuated *Salmonella* vaccine vector [11]. In recent years, use of live attenuated avirulent *Salmonella* vaccine vectors to deliver heterologous antigens has gained attention because of their ease of use in mass vaccination and their capacity to colonize gut-associated lymphoid tissues and systemic compartments effectively [15–17] and to induce protective systemic and mucosal cell-mediated and antibody-mediated immune responses against heterologous proteins [11,18–22]. Attenuated *Salmonella* as vaccine vectors for other pathogen antigens have also been successfully used in chickens in recent years [11,20,22]. The objectives of the current study were to assess the immunizing ability of three *C. perfringens* immunogens (PFOR, HP and FBA) when delivered orally through a *Salmonella* vaccine vector and also to identify B-cell epitopes in tPFOR and HP that protected birds against a severe NE challenge in a previous intramuscular immunization experiment to provide information about possible antibody recognition sites of these proteins.

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2. Materials and methods

2.1. Epitope mapping of HP and tPFOR

To identify B-cell epitopes of HP, a total of 169 peptides were synthesized (SPOTs synthesis) on a derivatized cellulose membrane (Sigma–Genosys Biotechnologies, Woodlands, TX). The synthesis of peptides was based on the 1020 amino acid sequence of HP such that synthetic peptides were 12 residues in length with sequential peptides having six residue overlaps [26]. The synthesized membranes containing the synthetic peptides were either probed immediately or stored at −20 °C until needed.

The membrane containing synthesized peptides were washed briefly with methanol and then with Tris-buffered saline (TBS) three times for 5 min and blocked overnight with the blocking buffer (Sigma–Genosys Biotechnologies) with 5% (w/v) sucrose. After blocking, the membranes were washed with TBST (50 mM Tris, pH 8.0, 136 mM NaCl, 2.7 mM KCl and 0.05% Tween-20) for 10 min and incubated with immune sera collected from HP-immunized and protected chickens from a previous study [10] at a dilution of 1:500 for 2 h at room temperature. Then membranes were washed twice with TBST for 10 min and incubated with goat anti-chicken immunoglobulin Y (IgY: heavy and light chains: Cedarlane Laboratories, Hornby, Ont., Canada) at 1:2000 dilution at room temperature for 1 h. After incubation, membranes were washed with TBST and the bound antibodies detected using the chemiluminiscent substrate CDP-Star (Applied Biosystems, Foster City, CA) and enhancer nitro-block II (Applied Biosystems), both diluted to 1:100 with 0.1 M Tris–HCl, 0.1 M NaCl, pH 9.5. The membrane was visualized using a Molecular Light Imager (Berthold, Bad Wildbad, Germany).

The quantified signal of each spot was determined as a relative percentage of a selected spot (peptide) that showed the highest reactivity (designated as 100%) using Win Light Software (Berthold) and the corresponding value was given after subtracting the background reactivity of spots developed to the sera from non-immunized, unprotected chickens collected in a previous study [10]. To optimize the appropriate serum dilution for optimum immunoreactivity, the membrane was re-used 3–4 times after regenerating the membrane, performed according to the manufacturer's instructions (Sigma–Genosys Laboratory). Spots showing strong binding intensities were traced back on the primary sequence of HP and immunoreactive regions (epitopes) were identified.

To identify B-cell epitopes of tPFOR, a total of 94 peptides were synthesized on derivatized cellulose membrane (Sigma–Genosys Biotechnologies) based on the 564 amino acid sequence of tPFOR such that synthetic peptides were 12 residues in length, with sequential peptides having six residue overlaps. Staining of membrane with immune serum and immuno-reactivity measurement was performed as described for HP.

Table 1
Bacterial strains and plasmids used in this study

Bacterial strains and plasmids	Relevant genotype or phenotype	Source
E. coli strain		
χ6097	F⁻ *ara* Δ(*pro-lac*) φ80*dlac*ZM15 *rps* L Δ*asd* A4 *thi*	R. Curtiss III
DH5α	*supE44 lacU169(80lacZM15) hsdR17 recA1 endA1 gyrA96 thi1relA1*	Stratagene
Salmonella enterica sv. *typhimurium* strain χ9241	Δ*pabA*, Δ*pabB*, Δ*asd*, Δ*ara*BAD, *relA*198:*ara*CP_{BAD} *lac*I	R. Curtiss III
Clostridium perfringens strain CP4	Wild-type, virulent NE isolate	Laboratory
Plasmids		
pYA3342	Asd⁺;pBR *ori*	R. Curtiss III
pYA3342-*fba*	Asd⁺, *fba*	This work
pYA3342-*tPFOR*	Asd⁺, *tPFOR*	This work
pYA3342-*tHP*	Asd⁺, *tHP*	This work

2.2. Bacterial strains and plasmids

Bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* χ6097, *Salmonella typhimurium* χ9241 and pYA3342 were kindly provided by R. Curtiss III (The Biodesign Institute, Arizona State University, Tempe, AZ). These bacterial strains have a chromosomal deletion of the aspartate B-semi-aldehyde dehydrogenase (*asd*) gene which is complemented by an *asd*⁺ plasmid (pYA3342) expressing heterologous genes [23], thus ensuring that the recombinant plasmid is stably maintained [24]. *E. coli* χ6097 is a derivative of DH5α which was used as an intermediate host to clone the genes of interest. *Salmonella* χ9241, an attenuated strain derived from UK-1 strain (*S. typhimurium* wild-type) has a chromosomal insertion of *lac*I gene under a BAD (*ara*) promoter that enables a regulated expression of recombinant protein. Thus, antigen expression can be controlled by addition of arabinose (0.2%) to the culture medium. In the absence of arabinose or *in vivo*, the trc promoter in the plasmid (pYA3342) enables constitutive expression of the recombinant protein.

E. coli χ6097 and *S. typhimurium* χ9241 harboring pYA3342 containing *C. perfringens* genes were grown in Luria-Bertani (LB) medium (Difco, Detroit, MI) and diaminopimelic acid (DAP, 100 μg/ml) was added to the medium when uncomplemented Δ*asd* strains were grown. Virulent *C. perfringens* (CP4) was used for challenge experiments and the growth conditions and challenge procedure are described later in this section.

2.3. Construction and cloning of C. perfringens genes in E. coli and in S. typhimurium

Several attempts to clone HP (3 kb) into pYA3342 were unsuccessful. Hence, based on the B-cell epitopes identified in HP, a region of 325 residues (≈1 kb) that contained strongly reactive epitopes, putatively the most antigenic part of the protein was cloned into pYA3342 and the resultant gene and its product is named as trun-

Table 2
Primers used to amplify genes cloned into pYA3342

Gene	Direction	Sequence (5′–3′)	Amplicon size (bp)
FBA	Forward	CCGC**GAATTC**ATGGCATTAGTTAACGCAAA	900
	Reverse	CCGC**GTCGAC**AGCTCTGTTTACTGAACCGA	
tPFOR	Forward	CCGC**GTCGAC**ATTAGAACCAGTTGGAGATA	1600
	Reverse	CCGC**CTGCAG**GAAGATCCATTGTGATCTCT	
tHP	Forward	CCGC**GAATTC**TTCTGGGGATTTGATAACTC	975
	Reverse	CCGC**CTGCAG**CTCCTCACCTAAAGCTAGTG	

Restriction sites (in bold letters): **GAATTC**: EcoRI; **GTCGAC**: SalI; **CTGCAG**: PstI.