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The use of quorum sensing to improve vaccine immune response

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ABSTRACT

Enterotoxigenic *Escherichia coli* (ETEC) infection is an important cause of diarrhea in both newborn and post-weaning pigs, it is also responsible for economic losses on farms worldwide. Vaccines that use ETEC virulence factors have been well documented, and several vaccines containing inactivated bacteria with protective antigens, or purified (isolated) antigens are available on the market. Vaccination of pregnant sows is widely seen as an effective strategy for the control of the disease. Yet these vaccines very often do not lead to efficient protection. In this study, we produced an ETEC bacterin with the use of quorum sensing (QS), and observed a significant expression of F4 adhesin, and heat-labile toxin (LT) in the cultures when compared to the controls. Mice, and pigs vaccinated with the QS bacterin demonstrated higher antibody titers against these antigens when compared with commercial and control bacterin. Our results suggest that the system might bring promising improvements in ETEC bacterin efficacy.

1. Introduction

Quorum sensing is a signaling system present among bacteria that involves auto-inducer substances (AIs). When critical concentrations of these substances are reached in the surroundings, corresponding to high cell densities or quorums, unified actions may be triggered that involve the whole population, this may include coordinated modulation of specific genes [1,2]. Several researchers [3,4] have used quorum signaling systems to describe bacterial pathogenicity. In mammals, the hormones epinephrine and norepinephrine have shown an effect similar to AI-3 in quorum sensing systems. Both AI-3 and catecholamines are recognized by the same receptor, and they both modulate virulence gene expression in enterohaemorrhagic *Escherichia coli* [5].

Enterotoxigenic *Escherichia coli* (ETEC) are a major cause of diarrhea in pigs and are responsible for significant economic losses worldwide [6]. The principal ETEC virulence factor is the fimbria, which mediates bacterial attachment to the host enterocyte allowing colonization and proliferation. The production of enterotoxins stimulates intestinal cell secretion of fluids and electrolytes leading to diarrhea [7,8]. Adherence is mediated by fimbrial structures, and porcine ETEC primarily express five types of fimbriae designated as F4 (K88), F5 (K99), F6 (987P), F41 and F18. F4 fimbriae are the most prevalent, and cause diarrhea and mortality in newborns, sucklings, and newly weaned piglets [9–11]. The infecting bacteria adhere to and colonize the intestinal epithelium, causing diarrhea through production of heat-labile and/or heat-stable enterotoxins (LT and ST respectively) [9,10].

Vaccination of pregnant sows has been widely adopted in order to give breastfeeding piglets passive immune response. Many vaccines employ recently developed technologies, using toxins, purified fimbriae, subunit proteins and DNA vaccines [12,13] however, conventional bacterins still play a major role in preventing colibacillosis of the swine. Nevertheless, bacterins may fail to contain the important protective antigens (*i.e.* F4 fimbriae) proteins.

In this study, we produced bacterin and used the quorum *sensing* signaling pathway to induce *in vitro* expression of important vaccine antigens, such as fimbriae and heat-labile toxin, and then investigated immune responses.

2. Materials and methods

2.1. E. coli and conditioned media

E. coli (ETEC) E68 (0141; K88ab; H4, Copenhagen Institute – Denmark) was kindly provided by Professor Dr. Carlos Gil Turnes (CDTec-UFPel). To prepare the conditioned medium, the strain was grown on blood agar at 37 °C for 24 h, and then isolated colonies were grown in Luria Bertani (LB) culture medium in flasks, and incubated in an orbital shaker (CERTOMAT® BS-T) at 37 °C for 7 h at 150 rpm. Afterwards, the culture was centrifuged at 13,000 × *g* for 20 min and the supernatant filtered (0.22 μm filter); the filtered supernatant was used as conditioner for the media. For bacterin production, the ETEC strain was cultured in an orbital shaker at

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37 °C, for 18 h, at 150 rpm, in LB medium, in 1000 mL flasks, containing 100 mL of culture medium. The cultures were of either LB culture medium (ConB) alone, or of LB media enriched by addition of 50% conditioned medium, with 500 μM epinephrine (Hipolabor). After the incubation period, samples were collected to verify purity (Gram stain, culture in Brain hart infusion agar media, BHA, Difco), and bacterial growth by measuring the number of CFUs per mL. The bacterial suspensions were inactivated by formalin at 0.2% for 24 h at 37 °C, inactivation was verified by inoculating the inactivated suspension onto BHA.

2.2. Bacterins

The bacterins were prepared using the cultures above mentioned with the bacteria number normalized to 2×10^9 CFU/mL^{-1}. Aluminum hydroxide 15% $Al(OH)_3$ was added as adjuvant.

2.3. Hemagglutination

In order to verify the expression of fimbriae protein, an adapted version of the hemagglutination (HA) technique proposed by Hovelius and March [14] was used. Briefly, the reaction was performed in 80-well U-bottom plates, the cultures (adjusted to an OD = 1.0) were diluted in saline (500 μL/well), and 500 μL of a 0.5% suspension of chicken erythrocytes in saline was added. The plate was then incubated at 4 °C for 2 h, and read.

2.4. Gene expression

Aliquots were taken from the ETEC cultures (QS and ConB), and from QS cultures where atenolol (β adrenergic receptor blocker, 2.000 μM, $C_{14}H_{22}N_2O_3$, Farmanostra) had been added. Total RNA was then extracted, and standardized at 1 ng for cDNA synthesis. cDNA synthesis was performed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The primers used in this experiment were designed using the program PRIMER 3 v. 0.4.0 following the GenBank access number AY437806 for *faeG*: forward CGCAGGTTCTTACAGGGAAA, and reverse GACCGTTTGCAATACCC AGT; AF450247 for *faeC*: forward CTGTTGATGCACAGGCTGAT, and reverse TAC TCTCCCGCCTTCACATC; M17873.1 for *eltB*: forward ACGGAGCTCCCCAGACTAT, and reverse AATGTTTCGCCGCTCTTAAA; and HM583969 for 16*S* gene RNA: forward AGGCCTTCGGGTTGTAAAGT, and reverse GTTAGCCGGTGCTTCTTCTG. Reactions were performed on an Applied Biosystems® 7300 Real-Time PCR System. Gene expression was determined by quantitative real-time polymerase chain reaction (qPCR). The relative amount of mRNA for each gene was determined by the comparative threshold cycle ($\Delta\Delta C_T$) method, which was standardized using the 16S RNA gene sequence.

2.5. Measurement of F4 and LT protein expression

To measure F4 protein expression, ELISA from the ETEC cultures of the different treatments (QS and ConB), being formalin inactivated, were used for antigens. The plates (Nunc) were sensitized with *E. coli* cultures (50 μL/well, 1×10^8 CFU/mL) in carbonate–bicarbonate buffer (pH 9.6) for 1 h at 37 °C. Rabbit anti-F4 serum, kindly provided by Professor Dr. Carlos Gil Turnes (CDTec-UFPel), (50 μL/well) diluted at 1:50 in PBS-T (phosphate buffer containing 0.5% Tween 20) was added to the plates, which were then incubated for 1 h at 37 °C. Then, anti-rabbit immunoglobulin peroxidase conjugate (DAKOPATTS A/S) diluted at 1:2000 in PBS-T was added, and incubation followed at 37 °C for 1 h. Next, the plates were washed 5 times in PBS-T, and 50 μL Ortho-23 Phenyllenediamine (OPD) substrate/chromagen was added and allowed to react for 15 min in the dark, at room temperature. Absorbencies were measured in a micro plate reader (MR 700 MICROPLATE READER) at 450 nm. For LT expression, the same approach was used, changing only the primary and secondary antibodies. As primary antibody, we used a mouse anti-cholera toxin antibody 1:5000 (Sigma), and as secondary, an anti-mouse peroxidase conjugate 1:2000 (DAKOPATTS A/S).

2.6. Animals and vaccination

2.6.1. Mice

Thirty Balb/C isogenic female, 21-day-old mice were randomly divided into three groups of ten animals each, and were subcutaneously vaccinated on days 0 and 14, with a 0.25 mL dose per animal (13 × 4.5 needle attached to a 1 mL syringe). Group 1 (QS), bacterin produced in conditioned medium with epinephrine, Group 2 (ConB) control bacterin, and Group 3 (control) an 0.85% saline with 15% $Al(OH)_3$ aluminum hydroxide added. The animals were kept in isolators under controlled temperature, and were given *ad libitum* access to food and water during the trial period. Blood samples were collected from the three groups, from the retro-orbital venous sinus (using a Pasteur pipette) every seven days during the experimental period. The animal serum was separated, tagged individually, and stored at −20 °C until analyzed. For the LT-ELISA, a pool of sera from days 1, 7, 14, 21, and 28 of the experiment were used from each group.

2.6.2. Gilts

The vaccination study was carried out in a commercial pig farm, and with no history of neonatal *E. coli* infections. The gilts were approximately 8 months old, and were artificially inseminated when demonstrating signs of being in heat. Pregnancy was confirmed by ultrasound examination before being selected for entry into the experiment. The six gilts were randomly divided into 2 groups, one receiving the QS bacterin and the other the ConB bacterin. As a commercial control vaccine group we used three random gilts from the same farm herd. The gilts of the QS and ConB groups were vaccinated intra muscularly in the neck (i.m.) with 2.5 mL (a 25 × 7 needle attached to a 3 mL syringe), 4 weeks before farrowing, and again 2 weeks later. Blood samples were collected (*vena cava cranialis*) from the three groups, on the first day of vaccination, at 2 weeks, and at delivery day, (using a 13 × 4.5 needle attached to a 1 mL syringe). The serum was separated, tagged individually and stored at −20 °C.

2.6.3. Offspring

Offspring from the vaccinated gilts (QS, and ConB), and the commercial vaccine (Comm) control were bled (blood sampled) at birth before receiving colostrum, and at 72 h after having begun to receive colostrum. The serum samples were collected, individually tagged, and stored at −20 °C until analyzed. In order to perform the assays, a random pool of eight offspring from each gilt was used, the number of piglets born per gilt ranged from 8 to 12.

2.6.4. Piglets

Thirty-six piglets at 35 days old were divided in three random groups of 12 animals, and vaccinated with the same vaccine, dose and *via* as used in the gilts. The animals were vaccinated and then revaccinated at 15 days from the first dose. Blood samples were collected from the three groups; on the first vaccination day, 2 weeks later, and at 21 days of experiment, the serum was then separated and stored at −20 °C.

This study was approved by the Animal Ethics Committee of UFPel under Process n. 23110.007699/2008-52.