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Evaluation of the Efficacy of Maternal Vaccination by Using Dominant Pathogenic *Escherichia coli* Isolates to Control Neonatal Diarrhea in Individual Swine Farm

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As the young mammal is unable to develop efficiently its own local immune responses in the first few days after birth, the passive mucosal protection of neonate piglets is dependent on the continuous supply until weaning of maternal immunoglobulin. To deep evaluate the efficacy of maternal vaccination in swine, in this research, virulence factor genes of prevalent *E. coli* isolates from diarrheic piglets were determined, and two dominant pathogenic *Escherichia coli* isolates were screened out and inactivated to use as antigens to vaccinate pregnant sows. The maternal immunity was evaluated based on the titers of antigen-specific milk antibodies, brush border cells adherence inhibition and the passive protective efficacy of newborn piglets. It was confirmed that maternal immunization could result 2 weeks in the appearance of F4- and F6- specific antibodies in the milk. In addition, the immune colostrum exhibited the ability to inhibit the binding of F4- and/or F6-positive *E. coli* to porcine brush borders, while the control samples without the blocking action. Furthermore, maternal vaccination could significantly reduce the diarrhea incidence rate from 55.5% to 15.2% and evidently postpone the disease time from day 6.32 to 12.29 in average.

Key words: Piglet; Neonatal diarrhea; Maternal vaccination.

Diarrhea continues to be the most economically important disease of piglet, especially occurred immediately after birth (neonatal diarrhea) (Holland, 1990; Nataro *et al.,* 1998; Zhang *et al.,* 2007). This disease can have an infectious or noninfectious etiology, and the infectious pathogen is the most important ones that frequently due to infection by one and/or the other pathogens, such as bacteria, viruses and parasites (Morin *et al.,* 1976; Moore *et al.,* 1991; Naciri *et al.,* 1999). Fortunately the vast majority of neonatal diarrhea is of bacterial origin and frequently due to infection by pathogenic *E. coli* (Nagy *et al.,* 1999 and 2005). For protection against pathogenic *E. coli* diarrhea, specific antibodies that inhibit bacterial adherence to receptors on the intestinal cells or neutralize enterotoxins are important (Haesebrouck *et al.,* 2004; Ofek *et al.,* 1990). Maternal vaccination in pregnant sows with the available vaccines or suitable antigens could results in stimulation of production of serum antibodies which are concentrated in colostrum (Brandtzaeg, 2003;

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Hanson *et al.,* 2000; Salmon, 1999 and 2000). While young piglets take up sufficient amounts of colostrum, the infection pressure will not be too high (Haesebrouck *et al.,* 2004).

In traditional concept, *Enterotoxigenic E. coli* (ETEC) is a frequent, important and global cause of severe, watery diarrhea in the newborn pigs within the first few days of birth (Nagy *et al.,* 1999 and 2005). Many ETEC strains could produce one or more fimbrial adhesins (such as F4, F5, F6 and F41) and two major classes of enterotoxins (heat-labile toxin and heat-stable toxins) (Gaastra *et al.,* 1982; Nair *et al.,* 1998; Sears *et al.,* 1996). The first event in the infection process by these ETEC strains is adherence of bacteria to porcine intestinal epithelial cells, which is mediated by adhesins to allow the production of diarrhea-causing toxins (Nataro, *et al.,* 1998). Furthermore, the pathogenicity islands (PAIs) has become fashionable, such as high-pathogenicity island (HPI) which was previously described in *Yersinia spp.* (Bearden *et al.,* 1998; Buchrieser *et al.,* 1998) and could horizontally transferred to other bacteria species of *Shigella*, *Citrobacter*, *Klebsiella* and *Escherichia* (Bach *et al.,* 2000; Karch *et al.,* 1999; Schubert *et al.,* 1998). It was also reported that some HPI-harboring *E. coli* isolates from patients and animals with diarrhea were closely correlated with clinical symptoms (Xu *et al.,* 2000; Cheng *et al.,* 2006).

Although the commercial vaccines have optimistically led to a massive reduction in the incidence of neonatal diarrhea, there is a dearth of research on the maternal vaccination by using dominant pathogenic *Escherichia coli* isolates. The aim of this research was to investigate the efficacy of maternal vaccination of inactivated dominant pathogenic *Escherichia coli* against a commercial vaccine, especially in a certain farm, and re-authenticate that passive maternal immunity should still be the optimistic strategy for protection against diarrhea of newborn piglets.

MATERIAL AND METHODS

Collection of diseased samples

Rectal swab samples were obtained from 40 live diarrheic piglets (5.10±1.86-day old) on a farm in Jiangsu province, China, and all the samples were collected within 36 h after diarrhea. Bring each test sample to a final volume of 500 µl with LB broth (10g tryptone, 10g NaCl, 5g yeast extract, H_2O to 1000 ml, pH 7.4) and store at -70°C before followed detection.

Rapid PCR detection of ETEC and/or HPI-harboring *E. coli* infection

Transfer 100 µl dilution of each rectal swab sample into separate tubes containing 2 ml of LB broth and grow the liquid cultures with vigorous agitation at 37°C for 6 h. Transfer 0.5 ml of the liquid cultures to labeled microcentrifuge tubes, and centrifuge at 12 000 g for 5 min. Discard the supernatants and resuspend each pellet in 200 µl of distilled water. After boiling for 10 min, the suspensions were chilled on ice for 5 min, and then centrifuged at 12 000 g at 4°C for an additional 5 min. Transfer each supernatant to fresh tube and store at 4°C. To detect ETEC and/or HPI-harboring *E. coli* in the liquid cultures of rectal swab samples, PCR assays were performed as described previously (Cheng *et al.,* 2006). All the reagents were purchased from Takara Biotechnology (Dalian) Co., Ltd. PCR products were separated by size by 1% agarose gel electrophoresis along with DL2000 DNA markers and visualized after staining with ethidium bromide on a UV transilluminator.

Isolation and determination of the virulence factor genes of ETEC and HPI-harboring *E. coli*

Following rapid detection of ETEC and/or HPI-harboring *E. coli* in the liquid cultures of rectal swab samples, all the positive samples were submitted to bacterial isolation by streaking on Mackonkey agar plates. Following incubation at 37°C overnight, twenty colonies were picked out from each sample and cultured on LB agar plates at 37°C for 24 h. All the isolates were submitted to PCR examination for the genes of enterotoxins (STa, STb and LTa), HPI and fimbria (F18, F4, F5, F6 and F41). The extraction of DNA templates and PCR assays were performed as described previously (Cheng *et al.,* 2005 and 2006). In addition, all the ETEC and/or HPI-harboring *E. coli* were serotyped by using uni-factor serum of O antigen of *E. coli* (purchased from China Inst. of Vet. Drug Control).

Preparation of antigen and parental vaccination of pregnant sows

Tow bacterial isolates were screened out

and each bacteria was all cultivated in separate vessels containing 100 ml TSB broth (17 g tryptone, 3 g soytone, 5 g NaCl, 2.5 g K_2HPO_4, H_2O to 1 000 ml, pH 7.4) and 100 ml Slanetz broth (20 g tryptone, 1 g glucose, 9 g NaCl, H_2O to 1 000 ml, pH 7.6) respectively (Cao, 1991). Following incubation at 37°C for 48 h under aerobic conditions, all the liquid cultures were transfer to labeled microcentrifuge tubes, and centrifuge at 4 000 g for 15 min. Discard the supernatants and resuspend each deposit in 20 ml of 0.85% saline. All the aliquots were mixed together, and then add formaldehyde to final concentration 0.8%; Vortexed vigorously and inactivated at 37°C for 7 days. Following confirmation that no organisms were alive, the bacterial suspension was centrifuged at 4 000 g for 15 min. The resultant pellets were washed three times with 0.85% saline and resuspended in the diluents (0.85% saline, 20% aluminum hydroxide, pH 7.2) at a final concentration of 3×10^9 bacteria/ml determined by optical density at 600 nm.

After safety test was performed in five mice (18~22 g) by subcutaneous injected with 0.5 ml inactivated bacteria and clinical observed for 10 consecutive days, fifteen F4 and F6-seronegative first pregnant sows (30-week old) from the aforementioned farm were used in the immunization experiments. The animals were separated into three groups: one experimental group and two control groups. The sows of the experimental group were immunized intramuscularly with the inactivated bacteria (2 ml/sow, administer in a minimum of four sites) at the time of 40 days before the expected date of confinement, and the animals of the control group I were treated with the *E. coli* commercial vaccine from a certain company in the same way, while the control group II were treated with the diluents (0.85% saline, 20% aluminum hydroxide, pH 7.2) as blank control. Twenty-five days latter, the 3 groups were boosted in the same way with the same inoculums used in the priming.

Detection of antigen -specific milk antibodies

Milk samples were collected at days 1, 2, 3, 7, 10 and 14 postpartum. All the samples were filtered to get rid of particles, and removed solidified fat via centrifugation at 12 000 g for 10 min to gain the whey (Huang *et al.,* 2008). All specimens were stored at -20°C until followed test.

To analyze the presence and regularity of antigen-specific milk antibodies in the whey samples, tube agglutination tests were performed. For each sample, a row of twelve tubes were set up and 0.5 ml 0.85% saline was added to each tube (except tube 1). Then add 0.5 ml of the whey sample to be tested to tubes 1 and 2. Mix the contents of tube 2 and perform doubling dilutions to tube 11 and then discard 0.5 ml instead of adding it to tube 12. Add 0.5 ml of the respective reference bacterial suspension (2×10^9 bacteria/ml) to each tube, incubate in a water-bath at 37°C for 4 h and then at room temperature overnight. ETEC strain C83600 ($F4^+$) and C83710 ($F6^+$) (China Inst. of Vet. Drug Control) were used to determine F4- and F6-specific antibody respectively. Agglutination of the suspension was positive result, while suspension remains turbid was negative. The endpoint titer was determined by using the dilution in the last tube which showed a positive reaction (Cao, 1991).

Inhibition of the adhesion of fimbriated bacteria to brush border by whey

The preparation of brush borders were performed as described previously (Baker *et al.,* 1997; Li *et al.,* 2007). Briefly, a 10 cm segment of jejunum collected from one 7-day old piglet from Jiangsu JiangQuHai pig farm, which was at a loss for high neonatal diarrhea incidence (45~50%). Cut open along its longitudinal axis, and cleaned with cold hypotonic EDTA solution (5 mmol/l EDTA, adjusted to pH 7.4 with 0.5 mmol/l Na_2CO_3). The brush border membrane vesicles were obtained by scraping the mucosal surface of the tissue with a glass microscope slide, and then placed in 50 ml cold hypotonic EDTA solution. After incubation for 30 min at 4°C, the solution was centrifuged at 1000 g for 10 min to pellet the cells. The pellets were suspended in 50 ml cold phosphate-buffered saline (PBS, pH 7.4) and centrifuged again under the same conditions. The pelleted brush border cells were resuspended in cold PBS and adjust the concentration to approximately 1×10^6 cell/ml determined by direct microscopic count.

Fimbriated bacterial strains used in brush border binding and inhibition assays included S079111 (O138:K88) and C83915 (O138:987P). Strains S079111 was cultured in TSB broth and

C83915 was cultured in Slanetz broth. The bacteria were then washed twice with PBS, and resuspended in PBS at an intended bacterial concentration of about 1×10^9 bacteria/ml determined by optical density at 600 nm.

The fimbriated bacteria (1 ml) was first mixed with 1 ml whey samples and incubated for 60 min at 37 °C, then centrifuged at 3 000 g for 10 min and subsequent washed with 2 ml PBS. The pelleted bacteria were resuspended in 1 ml PBS and subsequently mixed with 1 ml brush border cells. After incubated for 60 min at 37°C, the cells were centrifuged at 100 g for 10 min and washed twice times with 2 ml PBS to maximize the removal of the unattached bacteria. Brush border cells with bound bacteria were resuspended in 5 ml PBS, and 100 µl of this suspension was used to prepare microscope slides for checking the adhesion situation. Following air-drying at room temperature and Methylene blue staining, the slides were visualized on CIS-1000 Cell Image System (Shanghai Tanon Science & Technology Co., Ltd, China) by using a light microscope (Leica, 1000×). Over 50 epithelial cells were checked, and inhibition was considered when there were less than five bacteria (in average) adhering to the brush border membrane. Whey from unimmunized sows and PBS were used as control, and all experiments were performed in duplicate.

Investigation of diarrhea incidence rate and disease time suckling piglets

All the 3 groups' sows were initiated breastfeeding within 1 hour postpartum and continued 1 month. Diarrhea incidence rate and disease time of the suckling piglets were investigated until 15 days after birth.

Statistical analysis

The results were expressed as means ± standard deviations (SD). Statistical significance was determined by analysis of variance using SPSS for Windows.

RESULTS

ETEC and/or HPI-harboring *E. coli* infection in newborn diarrheic piglets

The specificity of the PCR assays were described previously (Cheng *et al.,* 2006). Among the 40 rectal swab samples from live diarrheic piglets, 25 (62.5%) samples only contained HPI-harboring *E. coli*, 2 (5%) samples just contained ETEC, 5 (12.5%) samples contained both ETEC and HPI-harboring *E. coli*, while 8 samples were not detected ETEC or HPI-harboring *E. coli*. The data suggested that at least 32 diarrheic piglets were infected with pathogenic *E. coli*. In addition, among the 7 samples which contained ETEC, 3 were LTa^+ and ST^+, 2 were STb^+, 1 was STa^+ and STb^+, while 1 was LTa^+, ST^+ and STb^+.

Virulence factor genes and O serotype of ETEC and/or HPI-harboring *E. coli*

Six hundred and forty bacteria isolates were picked from all the 32 rectal swab samples which contained HPI-harboring *E. coli* and/or ETEC, and the data (shown in table 1) of PCR examination determined that only 40 isolates (from 25 samples) were HPI-harboring *E. coli* and/or ETEC. Among the 40 *E. coli* isolates tested, 2 were $F4^+$, 3 were $F6^+$, while 5 were $F4^+$ and $F6^+$. No single $F5^+$, $F41^+$ or $F41^+$ was detected. In addition, all the 40 *E. coli* isolates were O serotyped, and the data show that the vast prevalent serotype was O138 accounting for 62.50% (21/40), followed by O65 (17.5%), O21 (10%), O139 (5%), O141 (5%), O139 (6.25%), O141 (5.00%), O9 (2.50%), O159 (2.5%) and O55 (2.5%). According to the aforementioned data, two pathogenic *E. coli* isolates (O138:K88, 987P:LTa,STa,STb and O138:K88, 987P:HPI) were screened out and inactivated to use as antigen in parental vaccination test.

The presence and regularity of antigen-specific milk antibodies

The presence and regularity of F4- and F6- specific antibodies were confirmed by tube agglutination test. The data show that intramuscular immunization of antenatal sows with the inactivated bacteria could resulted 2 weeks in the appearance of F4- and F6- specific milk antibodies (Table 2 and 3), and all had agglutination titers about 1 or2 log2 higher than that of the commercial vaccine group, while significantly higher than that of the blank control. In addition, the titers of antigen-specific milk antibodies were significantly higher in the first day postpartum, and then were continuous decreased.

The data are expressed as mean± SD (n=5). $P < 0.05$ (lowercase letters) was accepted

as significant differences and $P < 0.01$ (uppercase letters) was accepted as extreme significant differences among the groups.

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Effect of the whey on fimbria-mediated bacterial adhesion to brush border

Whey samples (endpoint titer 4, 2 and 0 log2 respectively) prepared from experimental group and control group II. The data of adherence inhibition assay confirmed that F4- and F6-specific antibodies exhibited the ability to inhibit binding of F4-positive (S079111) and F6-positive

Table 1. Summary of the virulence factors and O serotype in the 40 *E. coli* isolates from diarrheic piglets

Virulence factor O serotype	HPI	LTa	STa	STb	K88	987P	Numbers of isolates
O138		+	+	+	+	+	2
O138		+		+		+	1
O138				+	+	+	1
O138		+	+	+			3
O138		+		+			1
O138		+					1
O138	+			+	+		1
O138	+				+	+	1
O138	+					+	1
O138	+						9
O139	+					+	1
O139	+						1
O141	+						2
O65	+						7
O21	+						4
O9	+				+		1
O9	+						1
O159	+						1
O55	+				+	+	1
Total							40

Table 2. The presence and regularity of maternal F4-specific antibody in whey samples (log2)

Days postpartum Group	1d	2d	3d	7d	10d	14d
Experimental group	5.6±0.55 ^{A}	4.8±0.45 ^{A}	3.6±0.55 ^{A}	1.4±0.55 ^{a}	1.2±0.45 ^{a}	0.2±0.45 ^{a}
Control group I	3.0±0.71 ^{B}	2.4±0.55 ^{B}	1.8±0.45 ^{B}	0.8±0.45 ^{a}	0.2±0.45 ^{a}	0 ^{a}
Control group II	1.2±0.84 ^{C}	0.6±0.55 ^{C}	0 ^{C}	0 ^{b}	0 ^{b}	0 ^{a}

Table 3. The presence and regularity of maternal F6-specific antibody in whey samples (log2)

Days postpartum Group	1d	2d	3d	7d	10d	14d
Experimental group	8.2±0.84 ^{A}	7.6±0.55 ^{A}	7.0±0.71 ^{A}	5.0±0.71 ^{A}	4.2±0.84 ^{A}	2.4±0.89 ^{aA}
Control group I	6.6±0.55 ^{B}	6.2±0.84 ^{B}	5.6±0.55 ^{B}	3.6±0.55 ^{B}	1.8±0.45 ^{B}	1.2±0.45 ^{aAB}
Control group II	2.0±0.71 ^{C}	0.8±0.45 ^{C}	0.5±0.45 ^{C}	0 ^{C}	0 ^{C}	0 ^{bB}

Legends for Table 2 & 3

The data are expressed as mean± SD (n=5). P < 0.05 (lowercase letters) was accepted as significant differences and P < 0.01 (uppercase letters) was accepted as extreme significant differences among the groups.

(C83915) *E. coli* to porcine brush borders, while the control samples without the blocking action (Fig. 1 and 2). This indicates that maternal antibodies are more important and essential for reducing the risk of bacterial infection.

Diarrhea incidence rate and disease time of suckling piglets

All the piglets of the 3 groups were suckled within 1 hour and continued 1 month after birth. Diarrhea incidence rate and disease time (mean ± SD) were investigated from the 1st to 15th day. The data (Table 4) show that only 15.2% piglets of experimental group were suffered from diarrhea after the antepartum sows vaccinated with the antigens developed in this research, which was optimistic than the *E. coli* trivalent vaccine for newborn piglet from a certain company, and had statistical significant difference compared with normal saline treated group. Further more, most diarrhea cases of the experimental group were diseased approximately at 12th day, while the controls group I and II at about 9th and 6th day respectively. This suggested that passive mucosal immunity, acquired via colostrum, play important role in controlling neonatal diarrhea.

Table 4. Diarrhea incidence rate and disease time of the 3 groups

Group	Numbers of piglets	Numbers of diarrheic piglets	disease time (d) (incidence rate)
Experimental group	46	7 (15.2%) aA	12.29±1.38 A
Control group I	45	13 (28.9%) aAB	9.62±1.85 B
Control group II	45	25 (55.5%) bB	6.32±2.36 C

The disease time are expressed as mean± SD (Experimental group, n=7; Control group I, n=13; Control group II, n=25). $P < 0.05$ (lowercase letters) was accepted as significant differences and $P < 0.01$ (uppercase letters) was accepted as extreme significant differences among the groups.

DISCUSSIONS

ETEC is considered as an important and global cause of diarrhea in the newborn (suckling) piglets (Carniel *et al.,* 1992), but HPI-harboring *E. coli* is increasingly recognized closely correlated to clinical diarrhea (Fetherston *et al.,* 1994; Xu *et al.,* 2000; Cheng *et al.,* 2006). To control diarrheal diseases of piglets, we not only need to focus our efforts on improving environmental management, ensuring optimal nutrition and minimising stress, but also need to isolate and identify the pathogenic organisms. From an immunological point of view, passive mucosal immunity research include better insights in the prevalence of virulence antigen and better knowledge of immunity at the mucosal level.

Although the impact of the implementation of diarrhea control strategies has been optimistically in controlling the contagious pathogens and has led to a massive reduction in the incidence of neonatal diarrhea, the commercial vaccines do not guarantee complete protection from this disease. Sometimes the commercial vaccines are unsuitable for a certain individual farm, this is because the vast variety of pathogenic *Escherichia coli*. Therefore, perfect vaccine to effective against neonatal pathogenic *E. coli* diarrhea in a certain individual farm should be contained the prevalent bacterial antigens.

In this research, two dominant *E. coli* isolates were screened out from a farm, and the inactivated bacteria were used for maternal vaccination to control neonatal diarrhea. The results not only revealed that maternal immunization could result 2 weeks in the appearance of F4- and F6- specific mucosal antibodies in whey, but also provide further evidence for the important role of immune colostrum in controlling neonatal diarrhea. However, it was also found that the levels of F6-specific antibodies were greater than the F4; whether the different immunogenicity or different expression level in vitro were existed between F4 and F6 remain further research.

Fortunately, we also found that the efficacy of the antigens derived dominant pathogenic *E. coli* isolates were optimistic than

that of the commercial vaccine. This maybe because the 2 pathogenic *E. coli* isolates were all harbored HPI, but it is somewhat pity that the antibodies specific to the proteins coding by HPI were not determined. This mainly because of our knowledge is limited in selecting candidate protein(s) of HPI as available detection antigen(s). Despite of this lack, this research still testified and supported that maternal vaccination and breastfeeding new born piglets is an optimistic and viable strategy to control neonatal diarrhea in swine farm.

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