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Protection of neonatal calves against fatal enteric colibacillosis by administration of egg yolk powder from hens immunized with K99-piliated enterotoxigenic *Escherichia coli*

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SUMMARY

The protective effects of egg yolk powder prepared from hens vaccinated with heat-extracted antigens from K99-piliated enterotoxigenic *Escherichia coli* (ETEC) strain 431 were evaluated in a colostrum-fed calf model of ETEC-induced diarrhea caused by a heterologous strain (B44). The antibody powder was obtained by spray-drying the water-soluble protein fraction of egg yolks after removing the lipid and fatty components by precipitation with hydroxypropylmethylcellulose phthalate. A total of 16 colostrum-fed calves were studied to determine whether the orally administered antibody powder would prevent fatal bovine colibacillosis caused by a virulent ETEC strain. Clinical response of individual calves was monitored and evaluated in the context of these variables: fecal consistency score, intestinal colonization, weight loss, and mortality. Control calves that were treated with vehicle (milk with egg yolk powder from nonimmunized hens) had severe diarrhea and dehydration and died within 72 hours after infection was manifested. In contrast, calves fed milk containing egg yolk powder with antipili agglutinin titers of 1:800 and 1:1,600 had transient diarrhea, 100% survival, and good body weight gain during the course of the study. Results indicate that the orally administered egg yolk powder protected against ETEC-induced diarrhea in neonatal calves and that the protective components may have been the antibodies raised by vaccination of chickens against ETEC.

Enterotoxigenic *Escherichia coli* (ETEC) is the major cause of diarrhea and death in neonatal calves.[1-3] Previous studies have indicated that these organisms have at least 2 known virulence factors[4-7] :colonization factors that mediate adhesion to the epithelium, thus allowing rapid proliferation of ETEC in the small intestine; and enterotoxins, either heat-stable or heat-labile that cause diarrhea and dehydration. The K99 pilus antigen is one of the major adherence factors found on ETEC isolated from neonatal calves.[8-10]

The pilus has been widely used as vaccine antigen for control of enteric colibacillosis. Immunization of pregnant cows with purified pilus antigen conferred pilus-specific protection to their neonates against experimental challenge exposure.[11,12] In passive immunization experiments, oral administration of antibodies derived from serum or colostrum, or of monoclonal antibodies[13] has offered promising results in preventing the disease. However, it is costly to provide large amounts of antibodies by these means. Of particular veterinary interest is the use of chicken egg yolk immunoglobulins for prophylaxis and treatment of some infectious intestinal diseases. We used this type of antibody because of its potential therapeutic value and the advantage of cost-effective production. A method of extracting antibodies from egg yolk by use of hydroxypropylmethylcellulose phthalate (HPMCP) was established. This lipid precipitant was used in the production of antibodies because it is nontoxic and compatible with spray drying conditions. The objective of the study reported here was to evaluate the efficacy of semipurified chicken egg yolk immunoglobulins against ETEC-induced diarrhea (experimentally) in calves as an alternative agent for direct passive immunization.

Materials and Methods

Bacteria and growth conditions—Enterotoxigenic *Escherichia coli* strains 431 (0101:K30;K99;F41.NM,ST+) and B44 (09:K30;K99;F41:NM,ST+) were used in this study.[a] Bacteria were grown in Minca broth[14] at 37 C for 18 hours with shaking. Strain 431 was used for preparative extraction of pili. After incubation, the bacteria were harvested by centrifugation at 12,000 × g for 20 minutes and were suspended in 50 m*M* phosphate-buffered saline solution (PBSS, pH 7.0). The pili fraction was detached from the bacterial cells by heating at 60 C in a water bath for 30 minutes then centrifuging as described previously. The supernatant was passed through a 0.45-µm membrane filter to remove remaining whole cells. Pili

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concentration was measured by the Lowry method,[15] using bovine serum albumin as reference protein, and the desired pili amount was used for immunization.

Preparation of pilus antibody from egg yolk—Pili vaccine contained 1 mg of crude pili antigen in emulsion oil mixed with 5% mannide monooleate,[b] injected IM (breast muscles) in chickens. On the sixth and 14th weeks after the initial injection, booster inoculations were administered in similar manner and eggs were harvested 2 weeks later when high pilus antibody titer had been reached in the egg yolk on the basis of the results of plate agglutination testing. To maintain high antibody titer in the egg yolk, chickens were given booster inoculations regularly (every 2 months) after the first booster administration and eggs were collected every 2 weeks after booster inoculations. The yolk was carefully separated from the egg white and yolk membrane, and was diluted sevenfold with distilled water. A solution of 5% HPMCP[c] was added to the diluted egg yolk to make a 0.5% mixture for lipid precipitation. After standing at 25 C (room temperature) for 18 hours, the water-soluble, immunoglobulin-rich protein fraction was obtained and was passed through a 0.45-μm membrane filter, then was applied to a spray-dry machine[d] operated at air-inlet temperature of 140 C and air-outlet temperature of 72 C. The dried antibody powder was stored in a dessicator at room temperature until use.

Titration of antibodies—Antibody titer against the challenge strain was determined by use of microtitration plate agglutination and mannose-resistant (MR) hemagglutination-inhibition (HI). Inactivated strain B44 whole bacteria were used as the antigen in the agglutination test. The bacteria were suspended in PBSS after 18 hours of culture in Minca broth at 37 C, washed twice, and resuspended in 0.5% formol PBSS. After inactivation at 37 C overnight, aliquots of the bacterial suspension in PBSS were added to arrive at a desired optical density reading of 0.42 at 620 nm. The adjusted bacterial suspension (0.5-ml volume) was then added to an equal volume of serial twofold dilutions of 1% antibody powder solution. After overnight incubation at 4 C, pilus antibody titer was defined as the reciprocal of the highest dilution of antibody powder with observable agglutination.

The MR-HI test was used for detection of pilus-specific antibody activity in the powder. A 0.05-ml volume of crude pili antigen (8 hemagglutinating units) was added to an equal volume of serial twofold dilutions of 1% antibody powder in PBSS containing 0.1% mannose. After incubation for 1 hour at 37 C, a 0.3% suspension of sheep or guinea pig erythrocytes was mixed in equal volume, and incubation was overnight at 4 C. Reactions were read and HI titer was defined as the reciprocal of the highest dilution with HI.

Challenge exposure and clinical observations—Sixteen newborn Holstein calves from ETEC-free farms were used in this study, and each calf was fed immediately after birth with 2 L of colostrum obtained from its dam. The colostral anti-pili agglutinin titer varied from < 1:2 to 1:8 in the different colostrum pools. At 12 and 24 hours after birth, calves were fed milk (agglutinin titer < 1:2) to dilute and wash out remaining colostrum in the gastrointestinal tract. A commercially available ready-to-feed milk formula[e] was used and contained no additives. However, in the actual protection experiment, egg yolk powder from nonimmunized hens and antibody powder from the egg yolk of vaccinated hens were added to the milk as the treatment regimen for the control and treated groups, respectively. About 4 hours after the final suckling period, calves were orally challenge-exposed with a virulent strain of ETEC suspended in 500 ml of milk administered through a nursing bottle. The infective dose per calf contained 1.6×10^{11} viable virulent ETEC strain B44 organisms grown overnight in Minca broth. Before infection with ETEC was initiated, calves were randomly distributed to a control group (group 1) which was given milk without egg yolk antibodies, and to 3 antibody treatment groups (groups 2, 3, and 4), which were given milk containing pilus antibody titer of 1:400, 1:800, and 1:1,600 (0.03, 0.06, and 0.12 g of antibody powder), respectively. At 2 hours after ETEC administration, treated calves were given between 1 and 1.5 L of milk, in which the corresponding amounts of antibody powder were dissolved to give the desired antibody titer; each calf was fed 3 times/d for 7 days after infection. Control calves were fed milk containing normal egg yolk powder (agglutinin titer 1:8; 0.1 g of antibody powder). Clinical response of each calf was recorded throughout the experiment and was evaluated in terms of fecal consistency score, weight gain, and mortality. Fecal scoring was evaluated and given numerical scores as follows: 0 = normal, 1 = soft consistency, 2 = mild diarrhea, 3 = severe watery diarrhea. Identification of rectal and intestinal swab specimens from which the ETEC strain was isolated was done by culturing the swab specimens on trypticase soy agar,[f] with 5% defibrinated sheep blood. The K99 ETEC strain was detected by use of the slide agglutination test with K99 antiserum.[g]

Statistical analysis—The variance *t*-test was used to assess the statistical significance of differences in fecal consistency scores and percentage of body weight change, whereas the Fischer exact test was used to assess differences in mortality between treated and control calves.

Results

Antibody titers in the egg yolk and antibody powder—The use of HPMCP was effective for precipitation of yolk lipoprotein; the final supernatant resulting from the process had reduced lipid content comparable to that obtained by use of a different lipid-acting agent mentioned in a previous report.[16] The approximate yield of egg yolk powder from a single egg after spray-drying was 700 mg. Agglutination of piliated bacteria in the assay revealed antibody titer ranging from 1:1,024 to 1:2,048 in the egg yolks from immunized chickens and higher titer of 1:12,800/g of the antibody powder. The agglutination titer of egg yolk powder from nonimmunized hens was 1:80/g. In MR-HI test, titer obtained was 1:6,400 and 1:800 for erythrocytes from sheep and guinea pigs, respectively,

[b] Arlacel 80, Maine Biological Laboratories, Waterville, Me.
[c] Shinetsu Chemical Inc, Tokyo, Japan.
[d] Model L-12, Ohkawara Kakohki, Kanagawa, Japan.
[e] Morinaga Milk, Morinaga, Tokyo, Japan.
[f] Becton, Dickinson & Co, Cockeysville, Md.
[g] Denka Seiken, Tokyo, Japan

Table 1—Clinical response of calves to challenge exposure with enterotoxigenic *Escherichia coli* (ETEC) strain B44 and treatment with antibody powder of various titers

Group	Antibody titer	No. of calves with diarrhea/total No. of calves on day* 1	3	5	7	No. dead/ total No. (%)	Duration of diarrhea (d)†	Weight gain (%)‡
1	8	4/4(3.0)	1/1(3.0)	0/0	0/0	4/4(100)	2.0±0.7§	−9.7±2.9
2	400	4/4(2.8)	2/3(1.7)	1/3(1.0)	0/3(0.0)	1/4(25)	2.5±0.9	−3.9±4.7
3	800	4/4(2.3)	0/4(0.8)‖	0/4(0.0)	0/4(0.3)	0/4(0)¶	1.3±0.4	+1.1±0.3¶
4	1,600	4/4(2.3)	0/4(0.5)¶	0/4(0.3)	0/4(0.3)	0/4(0)¶	1.3±0.4	+1.9±0.7¶

* Data in parentheses are expressed as mean fecal consistency score. † Data are expressed as mean ± SD. ‡ Mean ± SD percentage of weight change on postchallenge-exposure day 3. § Diarrhea resulted in death of control calves. ‖ ($P < 0.05$). ¶ ($P < 0.01$).

Table 2—Isolation of ETEC strain B44 from rectal swab specimens and small intestinal specimens of calves

Group	Rectal swab specimens Day 1	3	5	7	Intestinal specimens Doudenum	Jejunum	Ileum
1	4/4	1/1	0	0	4/4	4/4	4/4
2	4/4	4/4	4/4	4/4	3/4	4/4	4/4
3	4/4	4/4	4/4	4/4	2/4	4/4	4/4
4	4/4	4/4	3/4	2/4	1/4	1/4	2/4

Data are expressed as No. of calves that were ETEC-positive/total No. of calves.

indicating considerable differences between the 2 types of erythrocytes used.

Clinical response of calves after challenge exposure—All calves of the control group (group 1), when exposed to K99+ *E coli* strain B44, developed severe watery diarrhea and later died on postchallenge exposure (PE) day 3 (Table 1). Difference in fecal consistency scores between the treated and control calves was significant ($P < 0.05$) on PE day 3. Dehydration developed rapidly after the onset of diarrhea, and mean change in body weight was $-9.7 \pm 2.9\%$ at time of death. On the contrary, calves of the antibody-treated groups were protected against severe diarrhea and mortality resulting from exposure to the virulent strain, except for 1 group-2 calf that did not survive infection. This calf developed severe diarrhea and eventually died on PE day 2 (weight loss of 11.9%). Although the other calves of group 2 survived the infection, diarrhea and dehydration resulted in weight loss of 3.9% on PE day 3. All the other calves in the 1:800 and 1:1,600 antibody titer-treated groups recovered from the disease. Diarrhea was temporary and was not accompanied by dehydration or weight loss in susceptible calves. Antibody titer of administered egg yolk powder was correlated with duration of severe diarrhea or weight loss in affected calves. Calves administered powder with 1:1,600 antibody titer manifested better recovery from the disease, compared with calves given powder of lower titer, indicating that the protection achieved was dose-dependent. In addition to better weight gain, fewer calves of this group were excreting the bacteria in the feces and harboring the ETEC organisms in the small intestine at the end of the study (Table 2).

Discussion

In this study, we evaluated efficacy of the antibody preparations derived from egg yolks of laying hens immunized with pili antigens against experimentally induced colibacillosis in newborn calves. Our results support use of chicken egg yolk immunoglobulins as an alternative method for direct passive immunization of newborn animals against infectious intestinal disorders, instead of colostrum, serum, and hydridoma-derived monoclonal antibodies. Those compounds have drawbacks when used for therapeutic purposes, especially involving a large number of animals to be treated. Significant protection was achieved involving all calves treated with the antibodies. Survival against ETEC-induced diarrhea was 100% when high titer of antibodies was used for treatment in calves (groups 3 and 4). These calves did not have severe diarrhea, and no calves died after oral challenge exposure with virulent B44 strain. On the contrary, all calves of the control group manifested severe signs of disease after infection with the challenge strain and succumbed to the disease, resulting in 100% mortality. The most satisfactory clinical response against challenge exposure was observed in calves treated with powder containing 1:800 and 1:1,600 antibody titers; severe diarrhea was prevented in these calves. The diarrhea was of mild intensity and transient, and calves were already manifesting increasing trend toward body weight gain on PE day 3 (Table 1). The minimal titer against fatal diarrheal disease may have been 1:800, but 1:1,600 was more effective in clearing the pathogenic bacteria from the small intestine, as can be seen from fewer calves harboring the organisms (Table 2). Thus, greater amount of antibodies is necessary to successfully prevent the ETEC organism from colonizing the intestinal epithelium.

The K99 and F41 pili (both present in ETEC strains 431 and B44) have been found to adhere to intestinal epithelial cells of calves,[17] and mannose-resistant hemagglutination of sheep and guinea pig erythrocytes has been observed.[18] The MR-HI titer of the antibody powder was 1:6,400 and 1:800 for the K99 pilus and F41 pilus, respectively, indicating dual specificity of the antibodies raised in egg yolks of chickens immunized with crude pili antigens. However, K99 antibody titer was 8 times higher than F41 antibody titer. The 0 somatic antigens differed between the 2 ETEC strains (strain B44, 09 and strain 431, 0101) and if contaminating antibodies against the 0 antigen were present in the powder, they would have different specificity and could have not had an appreciable role in protection. The antipilus agglutination titer of egg yolk powder from nonimmunized hens was considerably lower, and protective capacity was not observed in a protection experiment involving neonatal calves. On the other hand, the clinical response of calves treated with the antibody powder indicated a certain degree of acquired resistance against challenge exposure with virulent ETEC; thus, protection might have been conferred by these antibodies in the powder preparation. Similar results were reported on efficacy of chicken egg yolk immunoglobulins against fatal colibacillosis in pigs in which the antibody

fraction of the powder preparation had been removed by immunoabsorption, resulting in loss of the protective capacity of the preparation.[16] Results indicate that presence of antibodies, in particular, fimbrial antibodies, might have had a substantial role in protection because the egg yolk powder from nonimmunized hens (negligible antipilus activity) did not confer protection in the control calves.

In studies evaluating the protection afforded by vaccinating dams against ETEC, Acres et al[11] and Collins et al[19] documented susceptibility of calves from dams with colostral titer < 1:4 and protection in calves from dams with colostral titer > 1:4, on condition that calves were housed together with dams and were allowed to suckle and libitum. In our study, the minimal protective titer of the antibody powder may be 1:800 and, when the powder is dissolved in 1 L of milk, could actually be as low as 1:0.8. Despite the isolated environment where calves were reared on limited feeding schedules, minimal protective titer of antibodies obtained was lower in our study. This difference may be attributable to the different challenge strain, challenge-exposure system, and mode of treatment used in this study. Two possible explanations for this result are apparent. The affinity of antibodies derived from colostrum is different from that of antibodies obtained from egg yolk. It is believed that animals inoculated repeatedly with the same antigens develop a humoral immune response that yields antibodies with higher affinity for that particular antigen. The chickens used in the study were hyperimmunized with high content of pili antigens, and the final antibody preparation obtained had high titer against the immunizing antigens. These antibodies may have combined well with the bacterial pili and disrupted the colonization of the organisms in the small intestine. Also, IgG is often digested and inactivated by gastric juice. The HPMCP used to separate yolk lipids in our study has been used as an enteric coating substance for some drugs.[20-21] Apparently, HPMCP-coated drugs are resistant to gastric juice, and dissolution in intestinal fluid is pH-dependent. It is likely that our antibody powder coated with HPMCP, although not perfectly, may have been conferred enteric resistance properties against low pH, thereby allowing safe passage through the stomach and ensuring the ultimate release of functioning antibodies in the small intestine. Studies on the kinetics of HPMCP-treated antibodies in the gastrointestinal tract, and their affinity and activity against the bacterial pilus antigens in the target areas of the small intestine should be helpful in clarifying their role in protection.

In conclusion, results of this study indicate that egg yolk extract from immunized chickens has potential therapeutic value in controlling infectious intestinal diseases in mammals. Oral administration of the egg yolk powder to calves can serve as an important alternative approach to the already existing modes of treatment against colibacillosis in cattle herds exposed to K99+ ETEC in the field.

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