Maternal Milk Contains Antimicrobial Factors That Protect Young Rabbits from Enteropathogenic Escherichia coli Infection

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Enteropathogenic Escherichia coli (EPEC) colibacillosis represents a major cause of lethal diarrhea in young children in developing countries. EPEC strains also infect numerous mammal species and represent a major economical problem in rabbit industry. Protection against this pathogen is a challenging goal both in humans and in other mammal species. Despite a good knowledge of the pathogenicity mechanisms of EPEC, the intrinsic and environmental factors that control the expression of EPEC virulence in mammals remain unknown. Moreover, the exacerbated sensitivity of young mammals to EPEC infection is still unexplained. Our goal was to investigate if age or other factors, like milk consumption, could be determinants that trigger the disease. We used rabbits as an animal model to study the role of milk in the sensitivity to an EPEC infection. Weaned and suckling rabbits were orally inoculated with EPEC strain E22 (O103:H2;K−) at 28 days of age, and the evolution of the disease was investigated in the two groups. In addition, in order to better characterize the interactions between milk and EPEC, we determined in vitro bacterial growth and the abilities of EPEC cells to adhere to epithelial cells in the presence of milk. Our results demonstrate a protective role of milk in vivo in association with in vitro antibacterial activity. These effects are independent of the presence of specific anti-EPEC antibodies.

Colibacillosis is a frequent disease in European rabbitries. Depending on the geographical area, different serogroups of Escherichia coli strains are predominant (3, 8). In France, strains belonging to the O103 serogroup and to the rhamnose-negative biotype are largely dominant among pathogenic E. coli strains (8), and their high virulence leads to important economic losses in farms due to high mortality rates, growth retardation, and treatment costs. These strains induce severe and lethal diarrhea in rabbits upon oral inoculation with as little as 104 CFU (32). O103 rhamnose-negative strains are currently classified as enteropathogenic E. coli (EPEC)-like strains and represent strains similar to human EPEC strains in their virulence mechanisms and pathology (12, 38). Because EPEC strains isolated from humans do not cause diarrhea in animals, rabbit strains from an EPEC-like pathovar (REPEC) have been proposed as a model for the study of EPEC affecting both humans and animals (31). Indeed, the locus of enterocyte effacement of these REPEC strains is organized in similar clusters of genes homologous to those identified in human EPEC strains (12, 22, 34). As with human EPEC strains, the characteristic attaching/effacing lesion provoked by REPEC O103 strains is characterized by intimate adhesion to the cell in cup-like pedestals associated with a localized degeneration of host cell microvilli (1, 24, 34, 38).

However, REPEC strains differ from human EPEC strains in that they do not express bundle-forming pili or any other identifiable type 4-like fimbriae, responsible for the first loose attachment of bacteria to enterocytes (38). Instead, a majority of REPEC O103 isolates produces a specific fimbrial adhesin, termed adhesive factor/rabbit 2 (AF/R2), which is responsible for the adhesion to enterocytes and HeLa cell lines (30). This adhesin shares homology with the K88 fimbrial adhesin (16). Even if the etiological agent and its pathogenicity are now well known, many uncertainties still exist with regard to the intrinsic and environmental factors that control the expression of EPEC virulence in mammals. In rabbit production farms, weaned animals are more sensitive than suckling ones to infection with O103 E. coli strains, with 4- to 5-week-old pups being the most affected (23). In agreement with these observations, epidemiological studies with humans have shown that breast-feeding is protective against infections with EPEC strains (2). This has led to several hypotheses, including the presence of specific receptors to E. coli on the intestinal epithelium for a given period of age, the negative impact of weaning for intestinal balance, and a more or less specific protection by maternal milk mediated by antibodies and various other substances. One main problem is that predisposition factors that may affect the sensitivities of young mammals to EPEC infections, like age or nutritional status (suckling or weaned), are often confused in experimental or epidemiological studies. However, understanding of the mechanisms un-
derlying the sensitivities of young mammals to colibacillosis due to EPEC strains is essential to effectively battle this disease in the future. Indeed, applications not only can be found for rabbit production but also can be extended to human health, notably, in certain parts of the developing world where EPEC is known to be an important cause of children mortality (33).

In this study, our goal was to investigate if age was essential for the trigger of the disease or if other factors, like milk consumption, could be determinants. We studied the sensitivities of weaned and suckling rabbits to an infection with an EPEC strain known to preferentially affect 4- to 5-weeks-old rabbits. Weaned and suckling rabbits were experimentally inoculated with EPEC strain E22 (O103:H2:K+–) at 28 days of age, and the evolution of the disease was investigated in the two groups. In addition, we carried out in vitro tests to understand the role of milk in the expression of the disease. We determined bacterial growth and the abilities of EPEC cells to adhere to epithelial cells in the presence of milk. Our results show a protective role of milk in vivo in association with an in vitro antibacterial activity.

MATERIALS AND METHODS

Experimental infections. This experiment was carried out with PS Hyplus 19 × PS Hyplus 39 rabbits (Grimaud group) from a breeding unit free of E. coli contamination. Litters, normalized to nine kits, were weaned either at 21 days (W21 group, five litters) or 35 days of age (W35 group, five litters). The environmental temperature was maintained at 19 ± 3°C. The lighting schedule was 7:00 to 23:00 h with the lights on. From 14 days of age, young rabbits had access to an experimental feed (on an as-fed basis, crude protein, 17.3%; non digestible fibers, 34.3%; starch, 9.1%), whereas does were fed with a commercial feed. None of the diets was supplemented with antibiotics. Water and feed were provided ad libitum. At this time, the does were separated from their pups, except for suckling. The does were moved to new cages, whereas young rabbits remained in their birth cage until the end of the experiment (63 days of age), unless they were moved to new cages to respect density standards. Indeed, if more than five rabbits from the same litter reached 35 days of age, half of the litter was moved to another cage by alternating the weight ranks. Does were not inseminated during the suckling period. Animals were handled according to the care of animals in experimentation, in agreement with French national legislation (decree 2001-486, 06/06/2001), and the study received approval from the regional ethical committee of Midi-Pyrenees (France).

Bacterial strains. An EPEC strain belonging to the O103:H2:K+– serotype (strain E22) was used. This strain, described previously (4, 8, 30), does not metabolize rhamnose but produces AFG1 and AFG2 fimbriae positive. REPEC strain E22 was cultured overnight at 37°C with shaking in Luria-Bertani (LB) broth. Young strain E22 (strain E22) was used. This strain, described previously (4, 8, 30), does not metabolize rhamnose but produces AFG1 and AFG2 fimbriae positive. REPEC strain E22 was precultured for 24 h in Eagle minimum essential medium (MEM) supplemented with 25 mM HEPES (pH 7), 10% fetal calf serum (FCS; Gibco BRL), rhamnose (500 mM), and gentamicin (80 μg/ml) at 37°C in 5% CO2-95% air atmosphere. Strain pEGFP-E22 was precultured for 24 h in Penassay broth (antibiotic medium 3; Difco) supplemented with 25 μg/ml of carbenicillin. Incubation of the bacteria with the cells was realized in 500 μl of MEM buffer with 5% FCS and 1% mannose, with a starting inoculum of 20 μl (about 2 × 108 bacteria per well). To test the inhibitory capacity of adhesion of the serum, milk, or lactoserum of the does, 20 μl of strain pEGFP-E22 bacteria grown overnight (109 CFU/ml) in Penassay broth was preincubated with different amounts of serum, milk, or lactoserum for 1 h at room temperature, prior to incubation for 30 min with HeLa cells, as described previously (5). After 30 min of incubation of the cells with the bacteria at 37°C, the cells were washed three times with PBS. The cells were then incubated at 37°C in PBS containing 2 mM EDTA. The cells were harvested by PBSD, washed two times in PBS, and immediately acquired with a FacsCalibur instrument (Becton Dickinson). A total of 104 HeLa cells were acquired per sample. Quantification of adhesion was monitored by using Cellquest software (Becton Dickinson).

The percentage of adhesion was calculated by using the following formula: (percentage of pEGFP-E22-positive HeLa cells without serum, milk, or lactoserum)/percentage of pEGFP-E22-positive HeLa cells preincubated with serum, milk, or lactoserum)%

Tissue sampling for immunohistochemical studies. A litter of nine suckling New Zealand rabbits and a litter of weaned rabbits (group W21) were orally inoculated with 2 × 106 CFU of strain E22 at day 28 and housed as described above. The animals in each group were killed at 3 days postinfection by intravenous overdosing with sodium pentobarbital. Tissues from the distal ileum (10 cm from the ileo-cecal junction) were removed immediately after euthanasia. The tissues were fixed in 10% formaldehyde and embedded in paraffin. Transversal tissue sections (3 μm) were used for immunochemoanal staining. The slides were treated with hydrogen peroxide and an ABC kit (Vector, Burlingame, CA) to remove the background before staining. The bacteria fixed on the epidermis were labeled by using primary antibody in the laboratory by intradermal injection of a sheep with formalin-fixed whole E22 (4). To detect the fixation of antibodies, we used a biotinylated anti-sheep immunoglobulin antibody (Vector Laboratories). Anti-sheep immunoglobulin fixation was revealed with streptavidin coupled with peroxidase (Vector Laboratories) and revealed with diaminobenzidine (Sigma). All tissue samples were scored blindly by using light microscopy observations (Leica).
Statistical analysis. For each age, a designation of 0 or 1 was attributed to each rabbit if it was dead or alive, respectively. These data, which are considered Bernoulli variables, were submitted to a variance analysis (GLM procedure; SAS, 1999); the effect of weaning age on the survival rate was tested. For *E. coli* excretion, the results were expressed in log$_{10}$ CFU·g$^{-1}$ of sampled feces. Among the *E. coli* cells excreted, the proportion of the infectious strain corresponded to the number of colonies not metabolizing the rhamnose among those tested. The effect of the weaning age on total fecal excretion of *E. coli* was analyzed by the GLM procedure (SAS, 1999).

### RESULTS

**Suckling temporarily protects young rabbits from colibacillosis.** In the experiment performed to determine whether suckling temporarily protects young rabbits from colibacillosis, we took several precautions in the experimental setting. First, the does had to be seronegative for the strain of *E. coli* used here, to exclude any humoral passive protection for the young by the maternal milk. Second, the kits should not show any sign of previous contact with this strain. Thus, all does were tested for the presence of anti-LPS O103 antibodies 10 days before parturition and were found to be seronegative (data not shown). Their 24-day-old kits were also seronegative (28% of rabbits randomly tested), and no *E. coli* strains metabolizing the rhamnose were found in their fecal output (all cages were tested) (data not shown).

The two groups of young rabbits were inoculated 28 days after birth, and mortality was determined daily. The origin of each death was assessed by a combination of bacterial analysis of the cecal contents and necropsic examination. Colibacillosis was not responsible for the deaths of six rabbits: one rabbit from the W21 group and five rabbits from the W35 group. The data for these animals were removed from the study.

Our results indicate that after inoculation of E22, the mortality presented different patterns of evolution according to the nutritional status of the young rabbits (Fig. 1). Indeed, the first deaths were observed in the weaned group (group W21) from day 4 after experimental infection. In sharp contrast, no mortality due to colibacillosis was noticed in the suckling group (group W35) until 8 days postinfection (36 days of age), with the first death occurring only 1 day after weaning. At that time point, 50% of the group W21 rabbits were already dead. This mortality rate was reached only on day 45 in the W35 group. Moreover, the disease developed quickly in the W21 group, with the final survival rate being 20% 13 days after the first death occurred, whereas this evolution was slower for group W35 rabbits (mortality was observed over a period of 19 days). From 33 to 49 days of age, the survival rate of group W35 rabbits was higher than that of group W21 rabbits (P < 0.05). However, the difference in the final mortality rate between the two groups (36% for the W35 group versus 20% for the W21 group) was not statistically significant (P = 0.102).

As for mortality, the morbidity of the rabbits was recorded daily. The main clinical signs observed in rabbits during this study were prostration, weight loss, anorexia, and abundant aqueous diarrhea sometimes accompanied by dehydration symptoms. The same time lag in the pattern for mortality between the two weaning groups was noticed for the pattern of morbidity (Fig. 2). Thus, the peak of morbidity was observed at 32 days of age in the W21 group (close to 74% of diarrheic rabbits) but was observed at 42 days of age for rabbits in the W35 group (about 52% of morbid rabbits). Morbidity reached 100% for the whole period of the study, and all rabbits presented diarrhea symptoms in at least one clinical assessment, whatever their weaning group.

**Suckling temporarily limits fecal *E. coli* excretion.** To study the development of colibacillosis and to evaluate intestinal colonization and the potential elimination of pathogenic bacteria by rabbits, we aimed at controlling the level of fecal *E. coli* excretion. Fecal excretion of resident *E. coli* was unaffected by the weaning age before challenge, namely, 24 and 28 days of age (Fig. 3). After oral inoculation, group W21 rabbits excreted more pathogenic *E. coli* than group W35 rabbits until 38 days of age (3.4 log [P < 0.05], 2.4 log [P < 0.001], and 1.3 log [P = 0.053] at 31, 36, and 38 days of age, respectively). From 42 days onwards, the excretion patterns became similar between the two weaning groups and remained high until 49...
days of age (>10^6 CFU · g⁻¹ of feces). The profile of bacterial excretion clearly corresponded to the evolution of clinical signs in the two experimental groups. After day 49, the levels were maintained at 10^4 pathogenic E. coli · g⁻¹ until 59 days of age, whatever the weaning age, but with a high variability between rabbits. In the W35 group, this variability could be explained by the elimination of the inoculated strain from some cages (three cages on day 56 and eight cages on day 59). In the group W21 rabbits, this phenomenon may result from the low number of remaining cages (only four cages on day 42 and seven cages on day 35). Individual cecal enumeration of E. coli at 63 days of age revealed that about half of the survivors had eliminated EPEC, whatever their weaning age (4/8 rabbits in the W21 group and 6/11 rabbits in the W35 group).

**Bacterial adhesion to the ileal epithelium is strongly reduced in suckling rabbits.** We next wondered if the differential clinical evolution and the differential levels of fecal excretion between weaned and suckling rabbits were associated with differential ileal bacterial attachment and tissue lesions. A litter of weaned rabbits and one of the suckling rabbits were killed 3 days after oral inoculation with E22. Ileal tissues were sampled and stained with an anti-E22 serum, as described in Material and Methods.

In all weaned rabbits, we observed an important bacterial adhesion to the ileal epithelium. Bacteria almost entirely covered the epithelium, and we could detect the intimate attachment of bacteria to enterocytes, a typical lesion characteristic of EPEC (Fig. 4a). This strong bacterial adhesion was associated with a modification of the ileal architecture, characterized by the atrophy of the villi and the desquamation of the epithelium. At the time of sampling, all rabbits were prostrate and about 50% of them already presented with diarrhea.

In contrast, bacterial adhesion was greatly reduced in suckling rabbits. Among nine rabbits tested, eight exhibited rare
bacterial adhesion, with only scarce spots of bacteria detected (Fig. 4b). In addition, the epithelium was intact and no inflammatory lesions were detected. The villi remained long and thin. None of these rabbits presented with clinical signs at the time of sampling. The remaining rabbit presented a more important E22 intestinal colonization. About 30% of the ileal epithelium was covered by bacteria (Fig. 4c). The attachment of E22 was associated with an intermediate inflammatory response, characterized by a shortening and a widening of the villi; however, it was not associated with epithelial desquamation. Interestingly, this rabbit did not display any clinical signs.

These results confirm a protective effect of milk ingestion against colibacillosis. Indeed, we demonstrated a strong difference in bacterial attachment to the epithelium between suckling and weaned rabbits. In addition, this reduced adhesion was not associated with important intestinal lesions.

**Milk and lactoserum have bacteriostatic effects in vitro.** The in vivo results prompted us to characterize the protective effect of doe’s milk in vitro. In order to define a potential bacteriostatic or bactericidal effect of doe’s milk on REPEC growth, E22 bacteria grown in doe’s milk were cultured for 5 h in LB medium, milk, or lactoserum, as described in Materials and Methods. Our results showed the clear bacteriostatic effects of milk (2 log units of growth reduction compared with the growth in LB medium) and lactoserum (more than 3 log units of growth reduction compared with the growth in LB medium) (Fig. 5). Interestingly, the bacteriostatic effect of lactoserum was almost complete. Indeed, the growth rate was equal to only 1.3 after 5 h of culture. However, we could not demonstrate any bactericidal effect of milk or lactoserum in vitro.

Thus, the protective effect of suckling against REPEC for young rabbits seems to be associated with a capacity to limit bacterial growth in vitro.

**Milk and lactoserum inhibit bacterial adhesion in vitro.** We previously developed an in vitro test to quantify the capacity of rabbit sera to inhibit the AF/R2-dependent bacterial adhesion to HeLa cells (5). In order to assess whether the protective effect of doe’s milk could be linked to an inhibiting adhesion effect in vitro, we adapted this test for milk and lactoserum samples.

Milk was tested at different dilutions, and we observed a complete inhibition of bacterial adhesion up to a dilution of 1/4 (Fig. 6A and B). The effect became variable but still important at the dilution of 1/10 but insignificant at the dilution of 1/50. A similar pattern of inhibition of adhesion was found when these experiments were performed with lactoserum (Fig. 6C). Finally, we wondered whether the inhibition of adhesion was stable over time during lactation. Milk sampling was done on days 17, 24, and 30 after birth. For practical reasons, it was difficult to obtain milk before 2 weeks of lactation. For this experiment, lactoserum were tested at a dilution of 1/4. The capacity of inhibition of adhesion was observed for all the kinetic points tested, with an increase at the end of lactation (day 30), although the differences were not significant (Fig. 6D).

In order to ensure that this inhibition of adhesion was not due to the presence of anti-E22 antibodies in the females, the sera and lactoserum of the does were tested for anti-O103 antibodies by an enzyme-linked immunosorbent assay. All does tested were found to be negative (data not shown). In addition, sera were tested for their capacity to inhibit adhesion (without treatment and after the same treatment that was applied to the milk samples). None of the sera inhibited the adhesion of E22 to HeLa cells (Fig. 6B and data not shown).

Taken together, our results show that the protective effect of suckling against REPEC infection for young rabbits is associated with a capacity to inhibit bacterial adhesion to HeLa cells in vitro. These results suggest that doe’s milk contains one or several antimicrobial factors whose effects can be detected both in vivo and in vitro.

**DISCUSSION**

Here, we provide evidence showing that late weaning may be desirable to protect fattening rabbits against colibacillosis. Indeed, we have shown that suckling rabbits are protected against infection with an EPEC strain that belongs to the O103 serogroup and that is known to be highly virulent (32). When weaned rabbits are infected by using similar conditions (infection with a strain from the same serogroup and inoculation at the same dose and age), the first deaths classically occur 3 to 4 days after inoculation, and a high rate of mortality is reached in only a few days (4, 8, 17, 23). This corresponds to the pattern of evolution that we observed in the group W21 rabbits infected at 28 days. Thus, rabbits weaned early do not exhibit exacerbated sensitivity to this pathogenic strain compared to rabbits weaned just before the inoculation. It should be also noted that suckling rabbits presented an atypical pattern of evolution of colibacillosis. They were protected as long as they were suckling, but disease (clinical signs, mortality) started to develop as soon as the rabbits were weaned. Although the evolution of colibacillosis was less brutal and intense, it was then quite similar to that in rabbits weaned earlier but had a time lag compared to that in rabbits weaned earlier. These observations suggest that milk plays an essential role against the virulence expression of EPEC.

Several mechanisms of action, probably combined, may
thereby be suspected. First, the in vivo growth of bacteria may have been stopped or reduced. Some milk components could directly exert an antimicrobial effect against pathogenic E. coli or could indirectly create unfavorable environmental conditions for their growth by modifying the commensal flora, the pH, or bacterial substrates. This hypothesis seems likely, as suckling rabbits presented a lower level of excretion of pathogenic E. coli in their feces until weaning. Moreover, according to the in vitro tests that we carried out, it seems that a direct bacteriostatic effect of some milk substances is implicated. Second, milk could protect against diarrhea by limiting bacterial adhesion to enterocytes. Indeed, the pathogenesis of EPEC infection starts by a first step of loose adhesion between bacteria and enterocytes mediated by specific adhesins (13, 16). This hypothesis was confirmed by our results ex vivo that showed a reduction in the level of adhesion of E22 bacteria to the ileum of suckling rabbits compared with that in weaned pups. It was also consistent with our results obtained by using a recently developed assay of inhibition of AF/R2-dependent adhesion of EPEC to HeLa cells (5). This assay is based on the ability of pathogenic E. coli O103 to adhere to HeLa cells in vitro via the AF/R2 adhesin (30). In this study, inhibition of adhesion of E. coli to HeLa cells was demonstrated upon preincubation of the bacteria with milk and lactoserum as well as after preincubation of milk and lactoserum with HeLa cells before incubation of the bacteria with epithelial cells (data not shown). These data suggest that some milk components may prevent adhesion of the bacteria to enterocytes by acting on fixation sites from both the bacterial and the epithelial cell sides. Indeed, the first step of epithelial colonization by E. coli strains results from the connection between bacterial pili and surface receptors on host cells. For instance, sialoglycoproteins act as receptors for the AF/R1 pilus of the rabbit RDEC-1 E. coli strain (37), and glycosphingolipid and transferrin act as receptors for the adhesin K88 from some pig enterotoxigenic E. coli ETEC strains (20). The exact nature of the receptor specific for AF/R2 has not yet been identified.

Finally, some substances present in milk could interfere with the mechanisms leading to diarrhea, independently of bacterial colonization. Indeed, the strong inflammatory response induced by EPEC adhesion to the epithelium is at least partly responsible for diarrhea (9, 26, 33). Several studies have demonstrated the anti-inflammatory properties of human milk, which contains regulatory cytokines such as interleukin-10 and

FIG. 6. Milk and lactoserum inhibit bacterial adhesion on HeLa cells in vitro. (A) Milk samples of seven does were tested for their ability to inhibit bacterial adhesion on HeLa cells. The percentage of pEGFP-E22-positive HeLa cells was determined by flow cytometry by detecting the green fluorescent protein (gfp) expression of pEGFP-E22. Samplings were done at day 30 after birth. Dilution factors are indicated under each histogram bar. Data are means ± standard deviations. (B) Fluorescence-activated cell sorter histogram visualization of the inhibition of bacterial adhesion to HeLa cells obtained with lactoserum and serum of a doe at a dilution of 1/4. Similar results were obtained for six other does. Light black line, E22 and HeLa cells (E22 adhesion, 57.3%); heavy black line, E22 preincubated with lactoserum (E22 adhesion, 4.8%); spotted line, E22 preincubated with the corresponding serum (E22 adhesion, 52.7%). (C) The lactosera of seven does sampled at day 30 after birth were tested for their ability to inhibit bacterial adhesion on HeLa cells by flow cytometry. The dilution factors are indicated under each histogram bar. Data are means ± standard deviations. (D) The lactosera of four does were tested at different time points of lactation for their ability to inhibit bacterial adhesion on HeLa cells. All lactosera were tested at a 1/4 dilution. Data are means ± standard deviations.
transforming growth factor β. Indeed, these molecules have been implicated, for instance, in the protective role of milk in infant necrotizing colitis (14, 21). In addition, some healing properties of human milk have also been described, mainly in association with the presence of epidermal growth factor (14, 25, 35). Interestingly, it has been shown that oral complementation of young rabbits with epidermal growth factor prevents the appearance of REPEC-induced diarrhea (6).

The natural virtues of milk in protecting young mammals against numerous pathogenic viruses and bacteria have been known for many years (39, 43). A specific passive protection by maternal antibodies is often responsible for this phenomenon. However, in this study, the transmission of specific antibodies by maternal milk was not involved, since all does were seronegative for the O103 LPS. More recently, other substances present in milk have also been shown to have antimicrobial properties: defensins (19); lactoferrin (42) and its derivative, the lactoferricin (15); various enzymatic complexes like lactoperoxidase, xanthine oxidoreductase, and lysozyme (28, 36, 40); fucosylated oligosaccharides (10); and various fatty acids (18, 27, 41). It has been suggested that these components could inactivate pathogens by diverse mechanisms (growth inhibition, perturbation of adhesion) and may thereby act additively and/or synergistically (18). In humans, for instance, fucosylated oligosaccharides and lactoferrin have been shown to inhibit the localized adhesion of EPEC to HEP-2 and HeLa cells in vitro (10, 11). So far, the presence of these different components in doe’s milk has not been demonstrated, although it has been shown that doe’s milk is free from lactoferrin (29). It would thus be interesting to characterize the specific antimicrobial molecules present in doe’s milk.

In conclusion, our work offers interesting prospects for the identification of the milk components implicated in the control of colibacillosis and could lead to applications in the agrifood industry. The findings from this work could also be used in the future to protect children in developing countries against colibacillosis, a major cause of diarrhea.

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Vol. 14, 2007

EPEC COLIBACILLOSIS PROTECTION BY MOTHERLY MILK

591