# Passive protective effect of anti-K99 antibodies against enterotoxigenic *E.coli* infection in neonatal calves

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**Keywords:**
Calf diarrhea, colostrum, enterotoxigenic *Escherichia coli*, indirect ELISA, K99

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Received: 21 November 2017
Accepted: 13 February 2018

## Abstract:

**BACKGROUND:** Enterotoxigenic *Escherichia coli* (ETEC) is the main causative agent of neonatal calf diarrhea. Passive transfer of antibodies that are derived from the dams of the calves through their colostrum plays an important role in disease prevention. **OBJECTIVES:** To determine the presence of natural specific antibodies against K99 antigen in dam's serum and colostrum as well as in calf's serum and examine the association between bacteriological and serological aspects for ETEC K99+ and calf diarrhea. **METHODS:** A specific PCR assay was used to detect K99-positive *E. coli* in fecal samples from two groups of normal and diarrheic calves. Specific antibodies against K99 fimbriae in dam serum, colostrum and calf serum were studied using indirect ELISA. **RESULTS:** Significant differences were observed between the antibody titer in normal and diarrheic groups. Significant differences were observed between normal and diarrheic calves in their serum anti-K99 titers (p=0.0005), their dams (p=0.0005) and colostrum (p=0.001), in which anti-K99 titer in dam's serum and colostrum was higher in diarrheic group, but in calf's serum was higher in normal group. **CONCLUSIONS:** Although anti-K99 antibody levels in diarrheic calves were less than normal calves, there is no correlation between the level of these antibodies and disease occurrence. This study suggests that anti-K99 antibodies do not solely promote immunity to ETEC infections.

## Introduction

Diarrhea is an important frequently reported disease in neonatal calves due to several adverse effects including mortality, economic loss, treatment costs, and decreased growth rate (Cho and Yoon, 2014). As stated by National Animal Health Monitoring System (NAHMS), diarrhea is the cause of 57% of calf mortality, especially in calves less than one month old (Dairy, 2008). Among a variety of diarrheagenic agents such as viruses, protozoa, intestinal parasites and bacteria, (Dubreuil et al., 2016), enterotoxigenic *Escherichia coli* (ETEC) is the main cause of severe watery diarrhea during the first week of life (Nagy and Fekete, 2005). Several investigations reported

the high prevalence of calf diarrhea associated with ETEC around the world (Shams et al., 2012, Younis et al., 2009). A major factor impacting ETEC pathogenicity is the close adherence of the bacteria to the intestinal wall without tissue invasion, which is mediated by fimbriae, mainly K99 (Klemm and Schembri, 2000). Afterwards, heat stable toxins (STa) cause fluid and electrolyte hypersecretion through activation of intracellular guanylate cyclase (Fleckenstein et al., 2010).

The rapid proliferation of ETEC may lead to neonatal colibacillosis, septicemia or fatal enteritis. The course of the disease is rapid, from weakness, diarrhea, and dehydration to death in less than 24 hours. Antibiotics rarely affect the disease outcome, because their positive effects are observed at least three days after administration (Duse et al., 2015). Otherwise, before developing the antibodies by calf, the ingestion of colostrum during the first hours of life is compulsory for survival of neonates (Dubreuil et al., 2016).

During the period of susceptibility, specific antibodies block the fimbria receptor interaction (Nagy and Fekete, 1999). To protect the gastrointestinal tract of the calf against bacterial adhesion, newborn animals should gain an adequate amount of antibodies from dam colostrum (Constable et al., 2016). Passive immunization by using bovine colostrum is an area of high interested. The transmission of immunity from mother to offspring via colostrum was first mentioned by Ehrlich in 1892 (Silverstein, 1996). Early after birth, due to immediate changes in the ruminant intestine, the majority of absorption of colostral antibodies occurs during the first 24 hours of life (Xu, 1996). In the first 3 to 4 weeks of life, calves are able to secrete 25 to 30% of absorbed antibodies into the gastrointestinal tract (Barrington and Parish, 2001, Cortese, 2009). Cattle appear to naturally be able to elicit an immunological response to *E. coli* antigens and have specific antibodies in their colostrum (Figueiredo et al., 2004). Natural antibodies against some *E. coli* lipopolysaccharides and virulence factors have also been detected in non-vaccinated cattle serum and their colostrum (Vilte et al., 2008). On the other hand, besides the presence of natural antibodies, neonatal ETEC infections continue to be a significant clinical problem.

In non-immunized, naturally infected animals, the presence of antibodies against *E. coli* with titers of 640 IU had been shown in colostrum of non-vaccinated cows (Lissner et al., 1996). In Iran, the presence of anti-*E. coli* were evaluated in 84 diarrheic and 100 normal calves' sera under one month of age, by using direct ELISA for detection of anti-K99 *E. coli* (RABANI et al., 2007). The presence of anti-shiga-like toxin antibodies was also shown in 84% of colostra by capture ELISA (Pirro et al., 1995). The presence of antibodies against *E. coli* and shiga-like toxin has been shown in colostrum of non-vaccinated cows (Morshedi et al., 2010). A quantitative assay of specific antibody in colostral whey from both immunized and non-immunized dams has revealed that IgG, IgA, and IgM with anti-O (somatic) activity were present in whey of all dams tested, whereas a marked deficiency of IgA and IgM anti-K immunoglobulin was noted in the whey from controls (Wilson and Jutila, 1976). In the case of passive immunization against ETEC, there are few reports on oral administration of colostrum obtained from naturally infected cows. Detecting the natural and specific antibodies in the colostrum of individual cows might

be important in protection and could explain why some calves with sufficient total antibody concentration, are susceptible to ETEC infections. However, ETEC pathogenesis is determined by two virulence factors, the fimbriae and enterotoxins (Nagy and Fekete, 2005, Nguyen et al., 2011). The expression of K99 fimbriae (or F5) accounts for nearly all cases of ETEC infection found in newborn calves (Jay et al., 2004). Although non-vaccinated cow naturally has anti-K99 antibodies (Figueiredo et al., 2004), it is believed that immunity to ETEC infections can be achieved by enriching the colostral anti-K99 antibodies. Based on this idea, different vaccines (Bacterin and crude or purified fimbriae) have been developed. Nevertheless, no definitive data on the efficacy of these vaccines are available, and ETEC infections are still a significant clinical problem in farm animals in spite of extensive use of fimbriae-based vaccines (Moon and Bunn, 1993, Roy et al., 2010).

The aim of this study was to evaluate the role of natural maternally derived anti-K99 antibodies in preventing the colibacillosis. We particularly assessed the presence of natural specific antibodies against K99 fimberia in dam serum, colostrum and calf serum. Possible correlations between specific antibodies in serums and colostrum as well as the differences between two groups of normal and diarrheic calves were analyzed. The prevalence of ETEC K99+ isolates was determined and the possible associations between bacteriological and serological aspects for ETEC K99+, calf diarrhea and anti-K99 antibodies were investigated.

## Materials and Methods

**Samples collection:** Blood samples were collected after feeding colostrum, from two groups of animals including 100 normal and 94 diarrheic calves which were less than 5 days old. Blood and colostrum samples were also collected from all their dams. Allocation of groups was based on the presence or absence of diarrhea in calf, during the first week after birth. All samples were obtained from four Holstein dairy farms with the same management. The study was conducted at the four dairy farms located in the south of Tehran, Iran.

The blood samples were taken by syringe with a vacutainer tube, from tail vein of cows and jugular vein of calves, incubated for two hours in incubator at 37 ºC, and the sera were separated by 10 min centrifugation (1500 xg). All serum and colostrum samples were stored at -20 ºC and analyzed by ELISA for the specific IgG against K99 fimbriae.

**Detection of K99-positive *E. coli*:** Fecal samples were collected from 94 diarrheic calves. All the diarrheic calves were aged 3-5 days old and the samples were collected within 48 h after diarrhea. The samples were placed in sterile tubes and transferred to the laboratory in a cool box, within 3 h of collection. After overnight incubation in tryptic soy broth (TSB), samples were subcultured to MacConkey agar plates. Three lactose positive colonies from each sample were examined by polymerase chain reaction (PCR). Samples were also stored in potassium dichromate, mixed well and examined for the presence of Cryptosporidia oocysts using a standardized sucrose wet mount method.

Total DNA extraction by boiling method was used as a DNA template. Overnight cultures in 2 ml nutrient broth were centrifuged for 5 min at 2795 xg. The bacterial

pellet was resuspended in 200 µl of distilled water and boiled for 10 min. Tubes were centrifuged again, and the supernatant was used as template DNA. Amplification procedure was performed according to previously described protocol for detection of K99-positive strains (Franck et al., 1998). The following primers were used for the detection of K99 positive *E.coli*: F: 5'TATTATCTTAGGTGGTATGG3' and R: 5'GGTATCCTTTAGCAGCAGTATTTC3' were used. All the reagents were purchased from Cinnagene Co. The PCR mixture contained 2.5 µl of 10x PCR buffer (Mg2+ plus), 0.5 IU of Taq polymerase, 250 µM dNTPs, 1 µM of each primer, 2 µl of DNA template, and deionized water to a final volume of 25 µl. The condition for amplification consisted of 94 °C for 5 min for initial denaturation and 35 cycles at 94°C for 1 min (denaturation), 53 °C for 1 min (annealing) and 72 °C for 1 min 30 s (extension), followed by 72 °C for 10 min (final extension). PCR products were separated by 1% agarose gel electrophoresis and visualized after staining with ethidium bromide on a UV transilluminator.

**Expression and purification of recombinant K99 (fanC):** Expression procedure was carried out according to the standard method. Briefly, a 513 bp fragment of K99 fimbriae (fanC region) was constructed and subcloned in a pET28a plasmid. Then competent *E. coli* strains of DH5a were transformed by the recombinant pET28a-K99. The recombinant construct was confirmed by colony PCR and sequencing. Plasmid was extracted from positive colonies, purified and used for transformation of *E. coli* BL21 (DE3) cells. Induction of protein synthesis was performed with 1 mMof IPTG (isopropyl b-D-thiogalactoside) in a culture of bacteria. Bacteria were incubated at 37 °C for 16 h with shaking at 2.5 xg, centrifuged and lysed by a mixture of 5.0 mM of EDTA, 50 mM of Tris, and 8.0 M of urea (pH 8.0) for 18 h. After centrifuging (18 000 g, 30 min at 4 °C), the soluble proteins were purified by affinity chromatography on Ni-agarose beads (Qiagen, Dorking, UK). Recombinant protein expression was evaluated by western blotting procedure using mouse monoclonal *E. coli* K99 pilus antibody (Abcam, UK).

**Optimization of the ELISA tests:** Optimization of the test was conducted using a checkerboard titration. The purified fimbrial K99 (fanC) recombinant protein was used for titration, a 1:32 dilution of K99 recombinant protein (10µg/ml). In 96-well microplate, 50 µl of four-fold dilution series (1:2 to. 1:512) was coated overnight at 4 oC. Unbound antigens were removed by washing with PBS-T (PBS containing 0.05% [vol/vol] Tween-20). Then the wells were blocked by 100 µl PBS-Blotto (PBS containing 0.5 % [wt/vol] nonfat dry milk) for two hours. After two additional washing steps 50 µl of pooled serum was added to wells using two-fold dilution series (1:1.25 to 1:40) and left for 1 hour at 37 °C. The plate was then washed and 50 µl of horse radish peroxidase (HRP)-labeled goat anti-bovine IgG (AbD Serotec, Oxford, UK) was added to each well and left for 1 hour at 37 oC. After washing, TMB substrate was added and the mixture was incubated in dark place for 15 min at RT. The reaction was then stopped by the addition of 50 µl/well of stopper solution (1% SDS). Absorbance at 450 nm was measured using a microplate reader (Stat FAX 2000, Awareness Technology, Inc., USA). The same procedure was used for checker board titration of K99-ELISA using fimbrial K99 (fanC) re-

Table 1. Comparison of specific detected anti-K99 antibody levels between normal, diarrheic, and total groups. Values represent means ± SE.

| Antibody in animal | Antibody titer Normal group | Antibody titer Diarrheic group | Antibody titer Total | p value |
|---|---|---|---|---|
| Anti-K99 in dam serum | 80.70 ± 3.30 | 112.76 ± 2.27 | 96.82 ± 2.30 | 0.0005 |
| Anti-K99 in colostrum | 174.44 ± 7.09 | 198.76 ± 16.33 | 180.52 ± 6.73 | 0.001 |
| Anti-K99 in calf serum | 122.29 ± 2.51 | 104.31 ± 2.98 | 113.25 ± 2.05 | 0.0005 |

Table 2. Correlation and titer ratio of the levels of specific anti-K99 antibodies between dams' serum, colostrum and calves' serum in total group. * Correlation is significant at the 0.05 level (2-tailed). ** Correlation is significant at the 0.01 level (2-tailed).

| Anti-K99 in Dam serum in relation to: | R | Titer ratio | Anti-K99 in Colostrum in relation to: | r | Titer ratio | Anti-K99 in Calf serum in relation to: | R | Titer ratio |
|---|---|---|---|---|---|---|---|---|
| Anti-K99 in Colostrum | 0.808** | 0.53 | Anti-K99 In Dam serum | 0.808** | 1.86 | Anti-K99 in Dam serum | 0.760** | 1.16 |
| Anti-K99 in Calf serum | 0.760** | 0.85 | Anti-K99 in Calf serum | 0.825** | 1.59 | Anti-K99 in Colostrum | 0.825** | 0.62 |

combinant protein.

**Indirect ELISA:** Indirect ELISA was used to measure IgG antibodies against K99 fimbriae in serum and colostrum of cow and serum of its calf. Based on checker board titration, microtiter plates were coated overnight at 4 °C with 50µl of 1:32 dilution of K99 recombinant protein (10µg/ml). Plates were washed one time with PBS-T and non-specific binding sites were blocked with 100 µl of PBS-Blotto for two hours at room temperature. Plates were washed two times with PBS-T before adding 50 µl of serially diluted sera and colostrum. After incubation and washing with PBS-T, 50 µl horseradish peroxidase-labeled goat anti-bovine IgG was used as a secondary antibody and processed as described above. Antibody titers were expressed as the reciprocal of the highest dilution that gave a reading above the blank absorbance value.

**Data analysis:** Titer data are expressed directly for calculation of group means and standard errors of the means (SEM). The natural log (LN) titer data was used for between-group comparisons which were performed by one-way analysis of variance (ANOVA) using Fisher's protected least significant difference set to the 95% confidence level. Fisher's r-to-z conversion of correlation coefficients was used to obtain the p values in correlation analysis. Results of statistical analyses were considered significant if they produced values of P = 0.05. The computerized SPSS version 21 was the software that was used for calculations and statistical analyses of data.

## Results

The anti-K99 level detected in the serum of normal and diarrheic calves as well as serum and colostrum of their dams are summarized in Table 1. Significant differences were observed in serum specific anti-K99 titers between normal and diarrheic calves (p=0.0005), their dams (p=0.0005) and colostrum (p=0.001), in which anti-K99 titer in dam's serum and colostrum was higher in diarrheic group, but in calf calves' serum was higher in normal calves. There were no significant differences of anti-K99 levels between diarrheic calves with *E. coli* K99+ and other diarrheic calves.

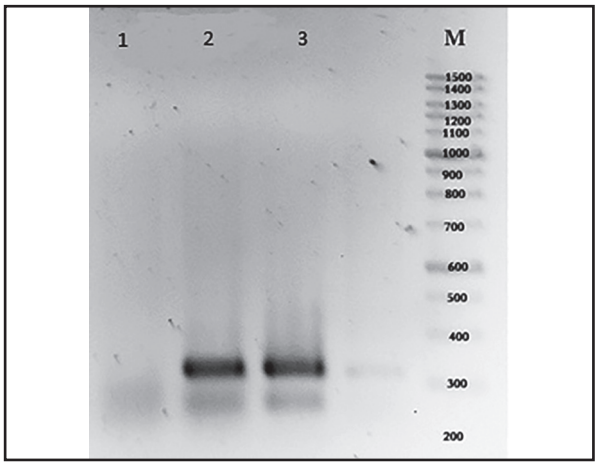


Figure 1. Ethidium bromide-stained agarose gel electrophoresis of PCR products (314 bp) for detection K99 of ETEC samples. Lane M: DNA ladder (100 bp Ladders, Fermentas, Germany); lanes 1: negative samples. lanes 2, 3 positive sample.

Regarding the correlation, there is a highly significant positive correlation of anti-K99 in dam serum with anti-K99 in colostrum (r = 0.808), and anti-K99 in calf serum (r = 0.760). Also, a highly significant positive correlation was found between anti-K99 in colostrum and calf serum (r= 0.825) (Table 2).

Isolated *E. coli* organisms from fecal samples were examined for the presence of K99 gene. A fragment of 314 bp was detected for K99-positive isolates (Fig.1). Based on PCR results, only isolates from six diarrheic calves out of 94 calves were K99 positive. All the positive cases were at the first week of age. All samples were negative for cryptosporidium oocysts.

## Discussion

Immunity to ETEC infections is mainly promoted by passive transfer of antibodies to neonatal calves. The enterotoxigenic E. coli, bearing the K99 attachment factor, can be found in calves younger than three days, especially in animals that did not receive any colostrum or received colostrum without any specific antibodies against this pathogen. The antibodies produced by cows in response to natural infection or vaccination are transmitted to the calf at birth via the colostrum (Constable et al., 2016).

The measurement of specific antibodies showed that the anti-K99 titers were higher in normal calves. One explanation is that the presence of anti-K99 antibodies lowered the prevalence of K99+ bacteria and diarrhea. Otherwise, if the level of antibodies is related to exposure to the bacteria (showed by PCR), the lower level of specific K99 in diarrheic calves could not be reasonable.

Anti-K99 titer is more in serum and colostrum of dams of diarrheic calves than that of normal calves while the titer of normal calves is more than that of diarrheic one (Table 1). This indicates that the normal calves had consumed more quantity of antibodies (6 liters of colostrum at the first 12 hours of birth, which represents 10% of the calf weight) than the diarrheic calves and the protection of the normal calves may belong to this difference.

Highly significant correlation between the level of anti-K99 antibodies in calf serum and colostrum (r = 0.825) and between colostrum and dam serum (r = 0.808) further confirm the impact of dam's immunity and its correlation with neonatal calves.

Our results are not in agreement with those reports, which declared that under natural conditions non-immunized cows have low levels of antibody to the ETEC and specifically its colonization factor (K99) (Contrepois and Girardeau, 1985). Nevertheless, our results further confirm the results of Smith et al. (1994) who believed that there are no differences in anti-K99 antibody levels between clinically healthy calves and those showing diarrhea

(Smith et al., 1994). In our experiment, although anti-K99 antibody levels in diarrheic group were less than normal group, there is no correlation between the level of these antibodies and disease occurrence. This could be confirmed by the observation of no significant differences of anti-K99 levels between diarrheic calves with *E. coli* K99+ and other diarrheic calves.

However, in the present study screening the fecal samples showed a low prevalence of *E. coli* K99+ in diarrheic calves. One explanation is that the dams were exposed to *E. coli* K99+ but the calves were infected with antigenically different types of bacteria. This may differ between different strains, different animal species, and different geographical areas (Vázquez et al., 2006).

The other main finding is the prevalence of *E. coli* K99+ in diarrheic calves which represents 6.3% (6/94). This is in agreement with studies conducted in Algeria (4.7%), India (3.86%), Iran (5.3%), Turkey (9.4%) and England (7.51%) (Akam et al., 2004, Içen et al., 2013, Kanwar et al., 2007, Shams et al., 2012, Sherwood et al., 1983). However, a higher prevalence was reported in Iran (14.1%), Iran (11%), Egypt (10.36 %), and India (20%) (Pourtaghi et al., 2013, Pourtaghi et al., 2015, Singh et al., 2007, Younis et al., 2009). On the contrary, a lower prevalence was recorded in America (0.57%) and Brazil (2.3%) (Salvadori et al., 2003, Zhang et al., 2007). The differences in the prevalence indicate variety distribution of ETEC in different geographical areas, and it may be due to variations in region, management conditions and hygienic measures. Our study represents that 100% of positive cases were at the first week of age, this is in agreement with Pourtaghi et al.'s (2013 and 2015) reports in Iran.

Finally, the protection of normal calves here may belong to antibodies against other virulence factors, or factors such as polyreactive antibodies, or factors other than immunoglobulins that protect the neonate and regulate the development of mucosal immunity (Gunti and Notkins, 2015). Balikci et al. (2014) mentioned that IgG concentration in healthy calves is more than that of diarrheic calves. Consumption of total antibodies led to protection of healthy calves against the disease (Balikci and Al, 2014).

We concluded that the prevalence of *E.coli* K99+ in diarrheic calves in the study region was 6.3 %, and the presence of anti-K99 antibody is not associated with the protection against the disease, because there are no significant differences between K99 positive and K99 negative cases. However, immunity to ETEC infections is not solely promoted by anti-K99 antibodies and may be attributed to other specific or nonspecific antibodies.

## Acknowledgments

This study was funded by the University of Tehran under Grant No. 30/6/7502015 and the colibacillosis vaccine project.

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مجله طب دامی ایران، ۱۳۹۷، دوره ۱۲، شماره ۲، ۱۰۶-۹۷

# نقش محافظتی پادتن‌های ضد K۹۹ بر ضد عفونت اشریشیا کلی انتروتوکسیژنیک در گوساله‌های تازه متولد شده

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(دریافت مقاله: ۳۰ آبان ماه ۱۳۹۶، پذیرش نهایی: ۲۴ بهمن ماه ۱۳۹۶)

## چکیده

**زمینه مطالعه:** اشریشیا کلی انتروتوکسیژنیک (ETEC) مسبب اصلی اسهال در گوساله‌های تازه متولد شده است. پادتن‌های مادری که بطور غیر فعال از طریق اغوز منتقل می‌شوند نقش مهمی در بروز بیماری و پیشرفت آن دارند. **هدف:** کاوش وجود پادتن طبیعی ضد K۹۹ در سرم مادران، آغوز و سرم گوساله ها و احتمال ارتباط نتایج باکتری شناسی و ایمنی شناسی ETEC K۹۹+ در ابتلای گوساله‌های تازه متولد شده به اسهال. **روش کار:** نمونه‌های مدفوع (سواب) از ۹۴ گوساله اسهال (۳-۵ روز سن داشتند) جمع آوری شد. نمونه‌ها در لوله‌های استریل قرار داده شدند و در یک جعبه ی سرد به آزمایشگاه انتقال یافتند. از روش PCR اختصاصی برای تشخیص وجودایکولی دارای فیمبریه K۹۹ در مدفوع گوساله‌های سالم و اسهالی استفاده شد و پادتن‌های اختصاصی طبیعی ضد فیمبریه K۹۹ در سرم مادر، آغوز و سرم گوساله با روش الیزای غیر مستقیم اندازه گیری شد. **نتایج:** تفاوت‌های معنی دار در عیار پادتنها در سرم گوساله‌های سالم و مبتلا به اسهال ($p=۰/۰۰۰۵$)، سرم مادران هر دو گروه ($p=۰/۰۰۰۵$)، وآغوز ( $p=۰/۰۰۱$)آنها مشاهده شد. عیار پادتن در سرم مادر گوساله‌های اسهالی وآغوز آنها بالاتر بود، اما در سرم گوساله‌های سالم از گوساله‌های مبتلا به اسهال بالاتر بود. **نتیجه‌گیری نهایی:** علی رغم اینکه سطح پادتن اختصاصی در گوساله‌های سالم بیشتر از گوساله‌های اسهالی است، هیچ همبستگی بین سطح این پادتن‌ها و وقوع بیماری وجود ندارد. این مطالعه نشان می‌دهد که ایمنی به عفونت‌های ETEC تنها توسط آنتی بادی ضد K۹۹ تأمین نمی‌شود.

**واژه‌های کلیدی:** اسهال گوساله، آغوز، اشریشیا کلی انتروتوکسیژنیک، الیزای غیر مستقیم، K۹۹

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