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Correlation between neonatal calf diarrhea and the level of maternally derived antibodies

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Summary

Passively acquired antibodies through colostrum will protect calves against etiological agents of neonatal calf diarrhea. Among them enteric diseases due to strains of Enterotoxigenic *Escherichia coli* (ETEC) are the most commonly occurring form of colibacillosis in newborn calves. Specific antibodies against whole ETEC cells and total immunoglobulin G in dam serum, colostrum and calf serum were determined. There were significant differences (P=0.0005) between antibody titers in normal and diarrheic groups, in which diarrheic group had a higher titer. Total IgG concentration in diarrheic calves (20.86 ± 0.49), their dams (23.48 ± 0.54) and colostrum (33.40 ± 0.50) was less than normal group (P=0.0005). There was a highly significant positive correlation between dam total IgG with calf total IgG (r=0.022; ratio=52.11). Colostral anti-*E. coli* antibody had a highly significant positive correlation with anti-*E. coli* in calf serum (r=0.345; ratio=0.62). Anti-*E. coli* antibody in calf serum had a highly significant negative correlati with total IgG of dam serum, colostrum and calf serum. While the level of anti-*E. coli* antibodies in diarrheic group was considerably higher than normal group, our findings reported here are in agreement that immunity to diarrhea also might be correlated with maternal cells or cellular components as well as cytokines which are transferred by colostrum to neonatal calves. Nevertheless, the level of maternally derived antibodies is a promising indicator for passive immunity and protection against diarrhea in neonatal calves.

Key words: Calf, Colostrum, Enterotoxigenic *Escherichia coli*, IgG

Introduction

Calf diarrhea is one of the most serious problems in the livestock industry and an important cause of economic losses in livestock due to high morbidity and mortality rate, high treatment costs and low growth rate (Osteras *et al*., 2007). The main causes of diarrhea in the first week after birth is *Escherichia coli*, especially Enterotoxigenic *Escherichia coli* (ETEC) strains, Cryptosporidium, Rotavirus and to a less extent Corona virus (Steiner *et al*., 1997). Enterotoxigenic *Escherichia coli* are the predominant pathogens of colibacillosis in calves, and are most frequent in the first four days of life (Lofstedt *et al*., 1999; Naylor, 2002). They attach to and colonize the intestinal epithelial cells, without invasion, and produce enterotoxins causing watery diarrhea in newborn calves (Foster and Smith, 2009).

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 become obvious at least three days after administration (Jacks *et al*., 1980). Otherwise, before developing the antibodies by calf, the ingestion of colostrum during the first hours of life is vital for survival of neonates (Bianchi *et al*., 1999). Failure of passive transfer (FPT) constitutes an economic, public health, and animal welfare issue because it is responsible for a higher level of disease, longer rearing period, and increased use of antimicrobials in calves (Earley and Fallon, 1998).

Different factors such as dam, calf immunity, and environment are involved in the occurrence of calf diarrhea (Cho and Yoon, 2014). Although there are several etiological agents for the disease, there is little difference between the patterns of prevention. Resistance of the calf to enteric disease is mainly related to calf immunity. To protect the calf against pathogens, newborn animals should gain an adequate amount of antibodies from dam colostrum (Radostits *et al*., 2017). The transmission of immunity from mother to offspring via colostrum was first mentioned by Ehrlich in 1892 (Silverstein, 1996). Soon after birth, due to immediate changes in the ruminant intestine, the majority of absorption of colostral antibodies occurs during the first 24 h of life (Xu *et al*., 1999). At the first 3 to 4 weeks of life, calves are able to secrete 25 to 30% of absorbed antibodies into the gastrointestinal tract (Banks and McGuire, 1989). In non-immunized, naturally infected animals, low levels of antibodies to the enteropathogens have been reported (Facon, 1995; Bogstedt, 1996).

Passive immune transfer not only depends on the level of immunoglobulin concentration in colostrum which is related to dam antibody production, but is also influenced by genetics, herd-level management, nutritional status and lactation number.

The level of maternal derived antibodies varies between calves. In the uniform condition calves may receive an adequate level of colostrum but vary in capability of colostrum absorption and secretion of antibodies to the lumen. For example, concentration of IgG in serum of neonatal calves is associated with polymorphisms at related genes or variation in hormonal regulation (Hurley and Theil, 2011). Detecting the variation in the level of antibodies might be important in protection and could explain why some calves with sufficient management are susceptible to diarrhea.

The objectives of this study were to: 1) evaluate the level of total antibodies and specific antibodies against whole germ bacteria (anti-*E. coli*) in calves' serum, their dam's serum and colostrum; 2) determine individual variations in the level of antibodies and making a comparison between the normal and diarrheic calves; 3) analysis of possible correlations between total IgG and specific antibodies in serum and colostrum of normal and diarrheic calves.

Materials and Methods

Samples collection

Blood was taken from 112 normal and 86 diarrheic calves less than 5 days old, after colostral uptake by them. The study was conducted at four dairy farms (average herd size = 10000 cows) located in the south of Tehran, Iran. In these herds dams had not received any vaccine during the 3 months before calving. Calves selection area was based on not born from dystocia, no signs of diseases other than diarrhea and no colostrum intake. There were similar regimens involving colostrum fed by bottle. Calves were fed colostrum within the first 12 h after birth with 10% of their body weight.

Allocation of groups was based on the presence or absence of diarrhea in calf, during the first week after birth. Blood and colostrum samples were also collected from all their dams. The blood samples were taken by syringe with a vacutainer tube, from jugular vein of calves, allowed to clot for 2 h in incubator at 37°C and the sera were separated by 10 min centrifugation (1500 × g). All serum and colostrum samples were stored at -20°C and analyzed by enzyme-linked immunosorbent assay (ELISA) for the total IgG and anti-*E. coli* content. A pooled serum from normal calves was used for Western blotting analysis.

Western blotting analysis

Natural antibodies against ETEC were tested by Western blotting analysis. The reference ETEC strain 510 and a K99 negative field strain were used as antigens. Proteins were separated using 14% sodium dodecyl sulfate polyacrylamide gel electrophoresis under reducing conditions and transferred onto a polyvinylidene fluoride membrane (0.45-mm pore size; Roche, Laboratories, Germany).

Then membrane was blocked with 5% nonfat dry milk in Tris-buffered saline (0.02 M Tris base-0.385 M NaCl-0.1%) and washed with Tris-buffered saline containing 0.05% Tween 20. Primary antibody (pooled serum), prepared from normal calf serums, was diluted in blocking solution (1/20), followed by incubation with secondary antibody (HRP-goat anti-bovine IgG). Color development was performed using α-chloronaphtol (Sigma, MO, USA) and TMB substrates in presence of H_2O_2.

Quantification of total IgG

Direct ELISA was used to measure total IgG antibodies in serums of calf, cow and colostrum. Microtiter plates were coated for 1 h with 50 µL of serum and colostrum samples that were diluted in PBS to the appropriate level (1:100000), along with serially (log_2) diluted purified bovine IgG (AbD Serotec, Oxford, UK) in PBS, ranging from 1-0.00781 µg/ml. The coating concentration of serum and purified bovine IgG were optimized to reach a linear regression. 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 h at 37°C. 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. Absorbance at 450 nm was measured using a microplate reader (Stat FAX 2000, Awareness Technology, Inc., USA). The standard curves were produced from purified bovine IgG which was included in all plates to minimize plate-to-plate variations.

Statistical analysis

The data represented by titer 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

Table 1 summarized the maternal serum and colostrum content of anti-*E. coli* and total IgG of normal and diarrheic groups, as well as their calves' serum. There is a significant difference between the specific anti-*E. coli* titers in normal and diarrheic calves serum (P=0.0005), as well as their dams serum (P=0.012) and colostrum (P=0.0005) (Fig. 1). Total IgG concentrations

Table 1: Comparison of total and specific IgG levels detected between normal and diarrheic groups. Values represent mean±SE

Antibody titer	Normal group	Diarrheic group	Total	P-value
Anti-*E. coli* in dam serum	1154.37 ± 23.12	1314.86 ± 59.63	1232.99 ± 31.93	0.012
Anti-*E. coli* in colostrum	1335.24 ± 31.22	1725.89 ± 33.47	1429.23 ± 28.90	0.001
Anti-*E. coli* in calf serum	1416.17 ± 28.31	3166.91 ± 103.57	2273.85 ± 81.59	0.001
Dam total IgG (µg/ml)	28.18 ± 0.77	23.48 ± 0.54	25.90 ± 0.50	0.001
Colostrum total IgG (µg/ml)	39.46 ± 0.70	33.40 ± 0.50	37.43 ± 0.45	0.001
Calf total IgG (µg/ml)	26.39 ± 0.48	20.86 ± 0.49	23.66 ± 0.39	0.001

Table 2: Comparison of specific IgG levels in relation to total IgG between normal and diarrheic groups. Values represent mean±SE

Antibody titer	Normal group	Diarrheic group	Total	P-value
Anti-*E. coli* in dam serum/dam total IgG	0.0479 ± 0.002	0.0577 ± 0.003	0.0522 ± 0.001	0.008
Anti-*E. coli* in colostrum/colostrum total IgG	0.0364 ± 0.001	0.0517 ± 0.001	0.0432 ± 0.001	0.001
Anti-*E. coli* in calf serum/calf total IgG	0.0671 ± 0.003	0.1682 ± 0.011	0.1117 ± 0.006	0.001

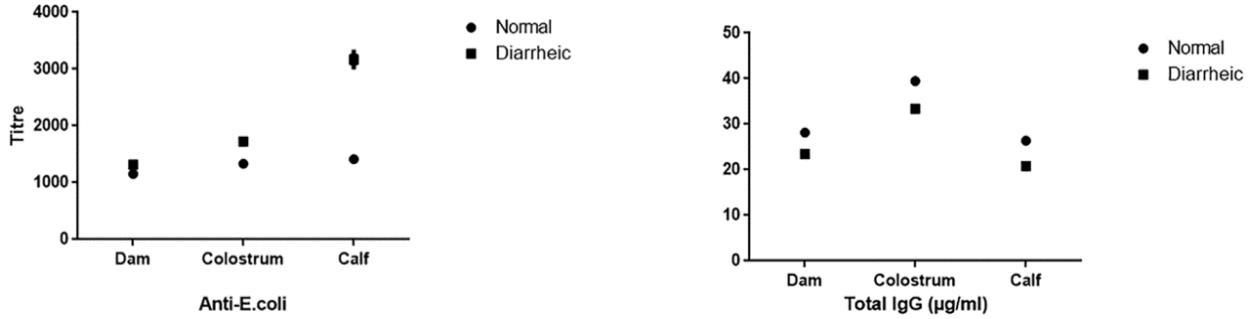

Fig. 1: Correlation of specific *E. coli* and total IgG antibodies between serum of dams, serum of calves and colostrum

significantly differ among the two groups of normal and diarrheic calves (P=0.0005), their dams (P=0.0005) and colostrum (P=0.0005). Diarrheic group had a higher titer of anti-*E. coli* antibodies than normal group. Total IgG concentration in diarrheic calves, their dams and colostrum was less than normal group (Fig. 1).

About the titer ratio of ANOVA in relation to total IgG, as shown in Table 2, there are significant differences between the specific anti-*E. coli* titers of normal and diarrheic calves (P=0.0005), their dams (P=0.0005) and colostrum (P=0.0005).

In Western blot assay, we used pooled serum prepared from normal calves (Fig. 2). The Western blot showed that the antiserum recognized ETEC K99$^+$ (510) and ETEC K99$^-$ bacteria. Western blotting of whole cell lysates showed that pooled serum from normal calves reacted with the number of ETEC proteins ranging from 20 to 50-kDa (Fig. 2, Lanes B-C). The band around 30-kDa was more intensely stained than other bands. A number of proteins revealed in SDS-PAGE were not recognized in Western blotting.

As indicated in Table 3, there is a highly significant positive correlation (P≤0.01) of dam total IgG with calf total IgG (r=0.303, P=0.001), dam anti-*E. coli* (r=0.239, P=0.001). Anti-*E. coli* antibody in colostrum had a highly significant positive correlation with anti-*E. coli* in calf serum (r=0.345, P=0.001). Anti-*E. coli* antibody in calf serum had a highly significant negative correlation (P≤0.01) with total IgG of dam serum (r=-0.232), colostrum (r=-0.295) and calf serum (r=-0.196).

According to the titer ratio in relation to total IgG, there is a highly significant positive correlation between anti-*E. coli* in dam serum with anti-*E. coli* in colostrum (r=0.212). A highly significant positive correlation was found between anti-*E. coli* in colostrum and anti-*E. coli* in calf serum (r=0.453, Table 4).

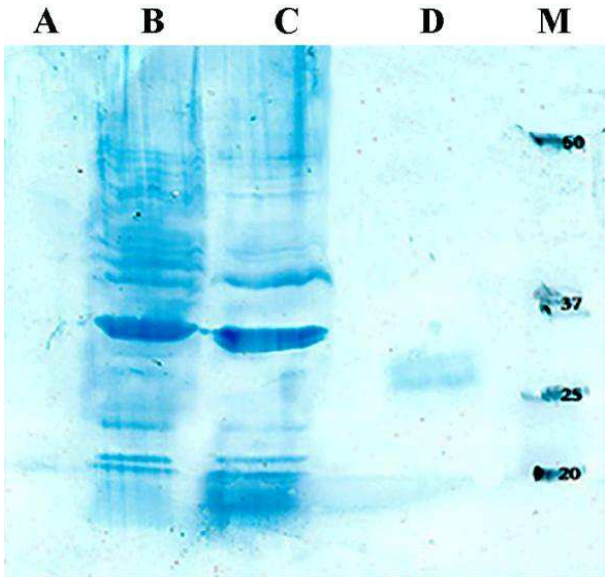

Fig. 2: Western blot of whole cell lysates of Enterotoxigenic *E. coli* bacteria probed with pool serum of calves. M: Molecular weight marker (10 kDa aprestain protein ladder). Lane A: *E. coli* strain 48A, K99$^-$, Lane B: *E. coli* strain 510, K99$^+$, Lane C: *E. coli* strain 134, K99$^+$, and Lane D: *E. coli* strain 78B, K99$^+$

Table 3: Correlation of the levels of total and specific antibodies between dams' serum, colostrum and calves serum

Anti-*E. coli* in dam serum in relation to	r	Titer ratio	Anti-*E. coli* in colostrums in relation to	r	Titer ratio	Anti-*E. coli* in calf serum in relation to	r	Titer ratio
Anti-*E. coli* in colostrum	0.082	0.86	Anti-*E. coli* in dam Serum	0.082	1.15	Anti-*E. coli* in dam serum	0.020	1.84
Anti-*E. coli* in calf serum	0.020	0.54	Anti-*E. coli* in calf Serum	0.345[b]	0.62	Anti-*E. coli* in colostrum	0.345[b]	1.59
Dam total IgG	0.239[b]	47.60	Dam total IgG	-0.128	55.18	Dam total IgG	-0.232[b]	87.79
Colostrum total IgG	0.037	32.94	Colostrum total IgG	-0.093	38.18	Colostrum total IgG	-0.295[b]	60.74
Calf total IgG	0.022	52.11	Calf total IgG	-0.134	60.40	Calf total IgG	-0.196[b]	96.10
Dam total IgG in relation to	r	Titer ratio	Colostrum total IgG in relation to	r	Titer ratio	Calf total IgG in relation to	r	Titer ratio
Anti-*E. coli* in dam serum	0.239[b]	0.02	Anti-*E. coli* in dam serum	0.037	0.03	Anti-*E. coli* in dam serum	0.022	0.01
Anti-*E. coli* in colostrum	-0.128	0.01	Anti-*E. coli* in colostrum	-0.093	0.02	Anti-*E. coli* in colostrum	-0.134	0.01
Anti-*E. coli* in calf serum	-0.232[b]	0.01	Anti-*E. coli* in calf serum	-0.295[b]	0.01	Anti-*E. coli* in calf serum	-0.196[b]	0.01
Colostrum total IgG	0.034	0.69	Dam total IgG	0.034	1.44	Dam total IgG	0.303[b]	0.91
Calf total IgG	0.303[b]	1.09	Calf total IgG	0.060	1.58	Colostrum total IgG	0.060	0.63

[a] Correlation is significant at the 0.05 level (2-tailed), and [b] Correlation is significant at the 0.01 level (2-tailed)

Table 4: Correlation of specific antibodies between serum of dams, serum of calves and colostrum in relation to total IgG (correlations for ratios)

Anti-*E. coli* in dam serum in relation to	r	Titer ratio	Anti-*E. coli* in colostrums in relation to	r	Titer ratio	Anti-*E. coli* in calf serum in relation to	r	Titer ratio
Anti-*E. coli* in colostrum	0.212[b]	1.208	Anti-*E. coli* in dam serum	0.212[b]	0.827	Anti-*E. coli* in dam serum	0.125	2.1398
Anti-*E. coli* in calf serum	0.125	0.467	Anti-*E. coli* in calf serum	0.453[b]	0.386	Anti-*E. coli* in colostrum	0.453[b]	2.5856

[a] Correlation is significant at the 0.05 level (2-tailed), and [b] Correlation is significant at the 0.01 level (2-tailed)

Discussion

Adequate transfer of maternal immunoglobulins is associated with health advantages by reducing pre- and post-weaning mortality due to infectious disease and increasing daily gain, feed efficiency, fertility, and milk production (Robison *et al*., 1988; DeNise *et al*., 1989; Wells *et al*., 1996). Immunity to ETEC infections is mainly promoted by passive transfer of antibodies to neonatal calves. Under natural conditions about 10-35% of calves may present FPT (Besser *et al*., 1991; Weaver *et al*., 2000). This demonstrates the importance of neonatal calf feeding and management. Identifying calves at risk for low serum IgG concentrations will assist dairy producers' efforts to ensure that calves receive adequate colostrum early in life. According to our results almost all of the normal and diarrheic calves did not present FPT (only 3 cases with less than 5 mg/ml). Positive correlations of colostral anti-*E. coli* with calf anti-*E coli*, further confirms the adequate passive transfer between dams and calves. In the population studied, the occurrence of disease which involved the passive immunity is not related to FPT and contributes to a more accurate evaluation.

Both groups of calves had anti-*E. coli* antibodies, as detected by Western blotting and anti-*E. coli* antibodies as detected by ELISA, demonstrating previous exposure to these agents. Western blotting showed moderate reactivity with the antigens of 18-50 kDa, and strong reactivity with proteins of 30 kDa. Three major protein bands with molecular weights of 18 kDa, 32 kDa and 39 kDa were detected on the basis of the serogroup assayed. Enterotoxigenic *Escherichia coli* was first grown under natural conditions known to support the production of virulence factors, including the heat-stable toxin and known colonization factors. Preliminary examination of subcellular protein fractions (concentrated culture supernatants) by immunoblotting with sera obtained from normal calves demonstrated multiple immunoreactive proteins, suggesting that many proteins are recognized during the exposure to ETEC (Fig. 2). Immunoreactive antigens in preparations correspond to a number of established or putative virulence proteins such as K99 fimbria which has a molecular weight of 18 kDa (Vazquez *et al*., 1996).

The measurement of total and specific antibodies showed that the serum IgG concentrations titers were higher in normal calves, but the anti-*E. coli* titers were higher in diarrheic calves. It is tempting to speculate that the disease might be related to the level of nonspecific antibodies, because in the diarrheic group only the total IgG level (dam serum, colostrum and calf serum) was significantly lower than the normal group. Our results are not in agreement with reports which declared that under natural conditions, non-immunized animals have low levels of antibody to the ETEC (Acres *et al*., 1982; Contrepois *et al*., 1985).

The absolute and relative value of anti-*E. coli* is more in diarrheic group than normal, while the total IgG is more in normal group (Tables 1 and 2). This indicates that the normal calves had consumed a greater quantity of antibodies than the diarrheic calves and the protection of the normal calves may belong to this difference, as mentioned by Klobasa *et al*. (1981) and Devillers *et al*. (2011) who said that with regard to immunity, IgG concentrations in plasma at 24 h of age are strongly correlated with colostrum intake (Klobasa *et al*., 1981; Devillers *et al*., 2011).

Our results indicated that the level of anti-*E. coli* in calf serum is correlated with colostrum anti-*E. coli* (r=0.354) but not correlated with dam serum. In addition, the level of anti-*E. coli* antibody in diarrheic calves is significantly higher than normal calves. This means that lacteal immunity against whole germ bacteria (anti-*E. coli*) has now been shown to be ineffective against diarrhea in suckling calves and there may be other antibodies responsible for protection (Contrepois and Girardeau, 1985; Bertschinger *et al*., 1990; Vazquez *et al*., 2006).

Regarding the total IgG, the titer of calf total IgG,

their dam, and colostrum in normal group is more than that of diarrheic group with a significant difference. This is in agreement with Balikci *et al*. (2014) who 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. This protection may belong to specific antibodies, polyreactive antibodies, or factors other than immunoglobulins that protect the neonate and regulate the development of mucosal immunity (Zhau *et al*., 2007; Gunti and Notkins, 2015). Polyreactive antibodies are a major component of the natural antibody repertoire, bind with low affinity to a variety of structurally unrelated antigens such as Gram-negative and Gram-positive bacteria and inhibit bacterial growth by lysis (Gunti and Notkins, 2015). The maternal immunity that the neonate always gets from dam is humoral immunity which includes specific systemic antibodies, and local mucosal antibodies. Cellular immunity, as mononuclear cells, has been shown to delay the onset of enteritis in the neonates (Cepica and Derbyshire, 1984). Factors other than immunoglobulins that protect the neonate and regulate the development of mucosal immunity with antimicrobial and/or immunomodulating activities, such as lactoferrin, transferrin, lysozyme, lactoperoxidase, milk mucins, free cytokines, chemokines, complement, lipids, carbohydrates, (Salmon *et al*., 2009). In spite of higher level of specific antibodies, diarrheic calves suffering from inadequate uptake of colostrum are prone to being affected by bacteria. Therefore, total content of colostrum which includes a higher concentration of humoral and cellular components, along with increasing the uptake of antibodies by the calf can reduce the incidence of diarrhea. Total IgG titers in calves could be a precious indicator for efficient passive transfer of all above mentioned factors from dams to their suckling offspring.

In conclusion, FPT was uncommon in herds of these farms. However, calves with lower serum IgG concentrations faced an increased chance of diarrhea. The higher total IgG in the normal calves had protected them from the disease, and the highly significant positive correlation between dam and calf confirms the adequate transfer of maternal immunity and protection against the disease. Level of antibodies against whole germ bacteria (anti-*E. coli*) in suckling calves has now been shown to not be related to the occurrence of diarrhea. This suggests that immunity to diarrhea also might be correlated with other pathogens, maternal cells or cellular components as well as cytokines which are transferred by colostrum to neonatal calves. Nevertheless, the level of maternally derived antibodies is a promising indicator for passive immunity and protection. As a highly significant positive correlation was found between dam total IgG with calf total IgG, it could be suggested that dam total antibody level is a suitable indicator for calf humoral immunity. Although other factors such as herd calf management should not be neglected.

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