



The immunobiology of calf scours  
by Richard Adam Wilson

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of  
DOCTOR OF PHILOSOPHY in Microbiology  
Montana State University  
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**Abstract:**

Passive immunity in experimentally induced bovine colibacillosis was studied in a 42 cow university herd. Pregnant dams were antigenized 1 to 9 weeks preparture with 2 injections administered by the subcutaneous and intramammary route with one of our vaccine preparations: killed bacteria (KB), live bacteria (LB), crude toxin (CT), or heat infusion broth (control).

The degree of scours (neonatal colibacillosis) induced by oral challenge of calves was evaluated clinically and reported by a semi-quantitative scour index as 0 to 4+.

Blood samples were obtained from the dams at day zero, at two weeks after the first injection and again at parturition which varied from 8 to 66 days after the second injection. Colostrum was obtained from the dams at parturition and 3 to 4 months post-partum. Blood samples were obtained from precolostral calves and again at 4 days of age.

Bacterial cultures were done on rectal swabs from calves at birth and at day 4. Blood cultures were randomly obtained from calves throughout the experiment.

Hemagglutinin and PHA titers were performed on sera and colostrum samples. A modification of the radial-diffusion technique was employed to measure the 4 immunoglobulins, IgG1, IgG2, IgA (S-IgA) and IgM as well as the specific activity (anti-O and anti-K) in colostrum and serum.

The data indicate that the neonates born to vaccinated dams were strongly protected against oral challenge with E. coli B-44. The hemagglutinin and PHA titers of colostrum directly reflected the efficacy of the vaccines. The effectiveness of the vaccine preparations was determined to be: KB>LB>CT>Control.

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Neonatal mice passively immunized with bovine colostrum whey were protected against oral challenge with E. coli B-44. Passive protection of infant mice with whey from control cows was related to the presence of colostrum IgG1 containing anti-K activity.

The data strongly suggest that immunity to scouring E. coli challenge was related to colostrum antibodies of the IgA and IgM class directed against the capsular antigen(s).

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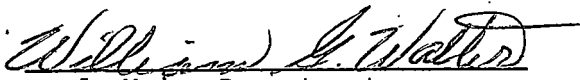
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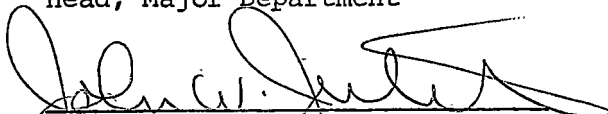
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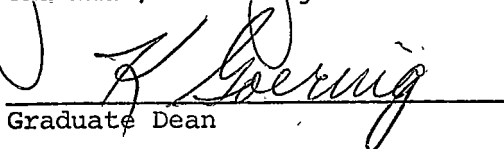
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Approved:

  
Head, Major Department

  
Chairman, Examining Committee

  
Graduate Dean

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It is stated without reservation that the relentless burdens of academic pursuit were wholly shared by my wife and four sons who carried more than their share without complaint or failure.

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## ABSTRACT

Passive immunity in experimentally induced bovine colibacillosis was studied in a 42 cow university herd. Pregnant dams were antigenized 1 to 9 weeks preparture with 2 injections administered by the subcutaneous and intramammary route with one of our vaccine preparations: killed bacteria (KB), live bacteria (LB), crude toxin (CT), or heat infusion broth (control).

The degree of scours (neonatal colibacillosis) induced by oral challenge of calves was evaluated clinically and reported by a semi-quantitative scour index as 0 to 4+.

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The data indicate that the neonates born to vaccinated dams were strongly protected against oral challenge with E. coli B-44. The hemagglutinin and PHA titers of colostrum directly reflected the efficacy of the vaccines. The effectiveness of the vaccine preparations was determined to be: KB>LB>CT>Control.

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The data strongly suggest that immunity to scouring E. coli challenge was related to colostral antibodies of the IgA and IgM class directed against the capsular antigen(s).

## INTRODUCTION

Calf scours is an acute, often fatal infection of young bovine and as a member of a large group of acute diarrheal diseases, presents a myriad of symptoms (signs) including diarrhea, dehydration, malaise, prostration and finally death. The epidemiology of the disease has been studied and defined by scores of investigators to yield etiologic agents classed within the bacterial and, more recently, viral groups. It has been known for nearly 60 years, however, that certain coliforms were commonly associated with a disease that may have a mortality rate as high as 66 percent. From available evidence, it appears that scours is caused primarily by a derangement in the equilibrium between the bovine neonate and certain bacteria in their environment. The relationship may possibly be a quantitative one, easily disturbed in favor of the multiplication of bacteria. Recent evidence suggests that the significant bacterium, the potentially pathogenic strains of E. coli, possesses certain pathogenic factors that may contribute to the disease process. The disturbance of the equilibrium may also be conditioned by an inadequate amount of colostrum which lacks certain immune factors to protect the neonatal calf from challenge.

Thus, calf scours represents a unique pathogenic complex that has not yielded to dissection by the bacteriologist, or serologist or clinician. The disease, in effect, represents an ecological problem that requires a bridging of disciplines and bodies of knowledge to

bring a measure of understanding into focus. A focus on relevant facts on the etiology, or more specifically, the immunobiology of calf scours yields the following information.

Escherichia coli is commonly isolated from the feces and the intestinal tract of neonatal calves (Smith and Orcutt, 1925; Smith, 1967; Mylria, 1969). There is adequate evidence that this organism can be the etiological agent of neonatal bovine colibacillosis (calf scours) (Gay, 1965) and of neonatal bovine colisepticemia (Aschaffenburg, 1949). Additionally, it is apparent that different strains are involved (Wramby, 1948; Smith, 1971; Gay, 1965; Cernea and Butura, 1965; Glantz and Jacks, 1969; Glantz, 1971) and also that there exists a relationship between smooth forms and pathogenesis (Gay, 1965).

Certain cellular constituents of E. coli have been examined for their role in pathogenicity and virulence in infectious intestinal disorders. Of particular importance are the O-somatic antigens, the K capsular antigen moieties and the enterotoxins produced by some strains of enteropathogenic E. coli. The O-somatic antigenic material may not be involved per se with the pathogenicity and virulence of E. coli (Gay, 1965). Most likely lipopolysaccharide (LPS), the endotoxin portion of the antigen, acts in concert with virulence factors associated with enteropathogenic coliforms.

There is evidence that would implicate K antigens in the virulence of E. coli (Glynn and Howard, 1970; Howard and Glynn, 1971). These studies indicated that K antigen reduces the complement sensitivity of E. coli by covering the antigenic determinants of the O antigen. It has also been shown that K antigen contributes to the adhesive qualities of certain strains of E. coli. Jones and Rutter (1972) showed that K-88 positive strains of E. coli adhered to the mucosa of the small intestine of neonatal piglets while K-88 negative strains did not. Adhesion of cell free K-88 antigen was also demonstrated. Additionally, studies by Smith and Linggood (1972) indicate that the common K antigen produced by calf and lamb enteropathogenic E. coli may function in the same manner as the K-88 antigens in the pig, that is, by facilitating adhesion to the intestinal epithelium. It is noteworthy that production of K-88 antigen by porcine strains (Ørskov and Ørskov, 1966) and the common K antigen produced by lamb and calf strains (Smith and Linggood, 1972) are under plasmid control.

Although the majority of the work on enterotoxin production by enteropathogenic E. coli has been done in swine (Gyles and Barnum, 1969; Gyles, 1971; Smith and Gyles, 1970), recent work reported by Smith and Halls (1968) and Smith and Linggood (1972) indicate that E. coli isolated from bovine are capable of producing enterotoxins. These findings are based upon studies using the intestinal loop assay

of De and Chatterjee (1963). Plasmid control of enterotoxin, an essential factor in enteropathogenicity of E. coli, has been shown by numerous investigators (Gyles, 1972).

A review of the literature indicates that the interpretation placed on the protective capacity of serum and colostrum against E. coli in neonatal bovine infections were predicated upon serological data (Briggs et al., 1951; Smith and Little, 1922; Ingram et al., 1953; Ingram et al., 1956; Gay, McKay and Barnum, 1964a,b,c). Other studies emphasized the relationship between the presence or absence of serum gamma globulins (Smith, 1967; Fey and Margarandt, 1962) and the incidence of colibacillosis and colisepticemia in neonatal bovine.

The more recent findings of Porter (1972) and Wilson et al. (1972) would indicate that certain classes of immunoglobulins, namely secretory IgA, may be involved in passive immunity of neonatal bovine. Hence, identifying specific immunoglobulin activity within various immunoglobulin classes may be a more valuable assessment of the protective capacity of immune colostrum. Their data further indicated that the quantity of immunoglobulins in calf sera generally reflected the dam's colostral immunoglobulin concentration. It would follow then that a critical evaluation of colostral-derived immunity against E. coli enteric diseases should include quantitation of antibody activity within the classes of immunoglobulins as well as of agglutinating activity of colostral and serum antibodies.

It has been demonstrated that in-situ production of secretory antibodies in colostrum follows intramammary instillation of antigenic material in rabbits (Genco and Taubmen, 1969), in goats (Pasioka et al., 1970) and bovine (Wilson et al., 1972) and in the ovine species (Lascelles and McDowell, 1970). A protective role for secretory immunoglobulins in infectious intestinal disorders is documented (Freter and Gangrosa, 1963; Ogra, 1969; Agarwahl and Ganguly, 1972).

Very recently, a limited clinical trial of immunization methods designed to elicit maximum passive immunity in suckling calves has been reported (Myers, Newman, Wilson and Catlin, 1973). In these studies it was shown that passive immunization of calves against experimentally induced scours was accomplished by vaccinating the dams. Interestingly, the vaccines were administered by the intramammary route as well as subcutaneously, prior to parturition. The test organism, E. coli B-44, caused severe diarrhea only in calves born to control dams while calves born to immunized dams were strongly protected.

This paper reports the immunological investigations of the cow-calf unit described in the earlier communication (Myers et al., 1973). To elucidate the protective factor(s) involved and to evaluate the immunological status of the cow-calf units, antibody titers and quantitative studies of total immunoglobulin levels and specific antibody activity in the various immunoglobulin classes were performed.

The findings of these studies and the results obtained with a murine model developed to evaluate "in-vivo" activity of specific anti-K antibodies are discussed.

## MATERIAL AND METHODS

### Test Animals

A 42 cow herd of pregnant Hereford dams was randomly distributed into 3 experimental groups of 9 cows each and one control group of 15 cows as described earlier (Myers et al., 1973). Each group was maintained in a separate outdoor pen, provided hay once daily and water ad libitum.

### Test Organism

The strain of Escherichia coli selected for his experiment was strain B-44 furnished by Dr. H. Williams Smith of Great Britain. E. coli B-44 was originally isolated from fecal material obtained from neonatal bovine exhibiting signs associated with the calf scour syndrome. This strain gave positive ligated gut loops in both the calf and rabbit. The serotype of this organism is O9:K.:NM.

E. coli B-44 is non-hemolytic, prototrophic, colicin positive, lactose positive and demonstrates both smooth-mucoid and rough non-mucoid colony morphology. Both the rough and the smooth colony types can be obtained on laboratory media. Viable organisms of both rough and smooth strains caused distention of the ligated loops in calf and rabbit intestine. However, the smooth-mucoid colony type was used in the production of crude toxin, live and killed vaccines as well as for challenge of the neonatal calves.

### Vaccines

The vaccines were described in a previous report (Myers et al., 1973). Briefly, B-44 was grown in brain heart infusion broth at 37°C with constant shaking. The live vaccine consisted of a 24 hour culture of the test organism which contained about  $3 \times 10^{10}$  viable cells per ml. The killed vaccine contained washed, formalinized (0.20% v/v) B-44 organisms at a concentration of approximately  $6 \times 10^{10}$  cells per ml. The crude toxin preparation was obtained by centrifugation and filtration of brain heart infusion broth culture supernatant that had been concentrated 100 fold by forced air evaporation at 15°C. The concentrated material was dialyzed against water at 4°C before formalin (0.20% v/v) was added.

Each vaccine was emulsified with an equal volume of mineral oil plus emulsifier prior to administration. The vaccine was given by the subcutaneous route and the intramammary route. A control vaccine consisted of brain heart infusion broth mixed with mineral oil and emulsifier.

### Vaccination Protocol

Each cow was given 5 ml. of antigen plus adjuvant subcutaneously and 4 ml. of vaccine by the intramammary route (1 ml. per quarter) twice within a 2 week interval. The first antigenation occurred

3 weeks before expected calving for the herd and the second dose was administered 2 weeks later.

#### Neonatal Challenge

The herd was kept under maximum surveillance to insure that the calves would not have free access to colostrum prior to acquisition of the first neonatal blood specimen. After parturition the calf was carried to the clinic and the dam was moved into a chute to facilitate collection of specimens. Blood specimens were taken from the calves via the jugular vein, and each calf was cultured by rectal swab. The dam was bled (20 ml.) from the jugular vein, and a sample of colostrum was taken from each quarter.

The cow-calf unit was then put into a small indoor pen to insure that the calf would obtain colostrum prior to challenge with test organisms at about 10 hours of age. The calves were challenged by gavage with 50 ml. of an 18 hour culture of E. coli B-44. Following the challenge, the cow-calf unit was moved to a common pen that maintained the challenged animals apart from the pregnant dams. The cow-calf units had access to shelter and dry bedding.

Time of onset, duration and severity of diarrhea (scored as 0, 1+, 2+, 3+, 4+) were determined for each calf (Table 1). Zero means no diarrhea was observed. The diarrhea of 1+ intensity was assigned if the calf was generally healthy and had normal rectal

temperature (approx. 38.9°C.), diarrhea, and no sign of dehydration. The 2+ diarrhea was scored if the calf had normal temperature, diarrhea, concavity of flanks, some dehydration as indicated by partial recession of the eyes, and slight apathy. The criteria of 3+ diarrhea were temperature higher than 37.8°C., diarrhea, moderate dehydration indicated by progression of recession of the eyes, and weakness (but calf retained ability to stand). The disease was scored 4+ if the calf's rectal temperature was less than 37.8°C. (often 34 to 36°C.), diarrhea, and severe dehydration indicated by decrease in normal skin pliability and marked recession of the eyes, was unable to stand, and perhaps was prostrate. Most of the calves were treated with electrolytes after reaching a 4+ diarrhea. Calves were removed from the experiment either after reaching a 4+ diarrhea or, in the case of calves that did not reach a 4+ diarrhea, 4 days after challenge inoculation.

#### Agglutination Studies

Bacterial agglutination studies were done according to the methods described in Campbell (1964). Somatic antigens designated as O antigens were prepared from an 18 hour culture of test organism in broth culture, centrifuged at 8000 rpm (9750 x G) for 10 minutes at 10°C. in a model RSB-2 Sorvall centrifuge. The packed cells were washed twice with sterile saline (0.87%) before boiling for 1 hour.

The boiled cells were washed with sterile saline before they were suspended at a final concentration of  $10^9$  organism per ml. (Nephelometric measurement). Titrations performed using B-44 cells boiled for 1 hour have subsequently been shown to include anti-K antibodies since 1 hour of boiling does not adequately remove all of the capsular material from this strain of E. coli.

External or capsular antigens, designated as K antigens, were prepared from an 18 hour culture of test organisms in broth, centrifuged and washed in the same manner described above. The washed, packed cells were suspended in formalin (0.20% v/v) and diluted to a final concentration of approximately  $10^9$  organism per ml.

When a new batch of either antigen was prepared, its serologic properties were compared with a standard batch of antigen to be certain the agglutination results using different lots were not significantly different. Difference in titers of new lots was always within a single dilution. Two fold serial dilutions of serum or colostral whey were pipetted for titration and after the addition of antigen, were incubated for 8 hours at 56°C. followed by a 12-14 hr. incubation at 37°C. The reactants were not centrifuged before the endpoint was determined. The last tube that demonstrated a one plus agglutination was considered to be the endpoint dilution for reporting antibody titer.

Passive hemagglutination experiments were performed by the method outlined in Campbell (1964). Endotoxin (LPS) was extracted from E. coli B-44 by the phenol water method of Westphal (1965). Sheep red blood cells (SRBC) were sensitized to B-44 LPS according to the technique of Neter et al. (1956). Sensitized SRBC's suspended in 1% normal rabbit serum were added to colostral whey or sera that were serially diluted in phosphate buffered saline. The tubes were incubated at room temperature for 2 hours then placed at 4°C. for an additional 12-14 hours. Following centrifugation at 1500-2000 x G., the cells were dislodged from the bottom of the serology tube and observed for agglutination.

#### Blood Samples

On the first day of the experiment, just prior to the administration of antigen, a blood sample was taken from each cow via the jugular vein. Subsequent blood samples were obtained at the time of calving and three months post-partum. Blood samples were obtained from each calf by jugular vein venipuncture both at the time of birth and again at four days of age. The blood was held at room temperature for one hour before the clots were dislodged from the tube walls and then separated from the serum by centrifugation. Sera were stored at - 20°C. until tested. All sera were heated at 56°C. for 30 minutes immediately before titrations were performed.

### Whey Samples

Colostrum or milk samples were treated with renin (stock solution diluted 1/300) and calcium chloride in a concentration sufficient to create a firm clot at 42°C. Anti-O and anti-K titers were performed on unheated whey samples and whey samples that had been heated for 30 minutes at 56°C. Passive hemagglutination titrations were done using heated whey only, since earlier studies in this laboratory indicated an insignificant difference in titers before and after heat treatment.

### Immunoglobulin Preparation

Bovine secretory IgA was isolated from saliva and colostrum. Approximately 100 ml. of saliva was obtained by suctioning the buccal cavity of 3 cows. The saliva was pooled and large particulate material was removed by centrifugation. The supernatant material was dialyzed in the cold against 0.03 molar ammonium chloride until the mucoid material was solubilized. The precipitate that formed during dialysis was removed by centrifugation. The remaining supernatant was dialyzed against three changes of phosphate buffered saline (PBS) for 48 hours. The colloidal suspension was concentrated three-fold by pressure dialysis in preparation for chromatographic separation. Ten ml. of the concentrated solution was added to a 2.5 x 40 cm. Sephadex G-200 column equilibrated with PBS. Three ml. fractions of eluate were collected with a Gibson fraction collector at a rate of 60 drops per minute.

Presence of protein in the fractions was determined by measuring the absorbance at 278 nanometers in a Coleman Hitachi Spectrophotometer. An elution curve was plotted from the absorbance data. The fractions that comprised the void volume were pooled, concentrated and passed again through Sephadex G-200. Two very distinct peaks were noted after the second passage. The first peak to come off the column was concentrated to 5 ml. and tested in an Ouchterlony double diffusion system against S-IgA antibody (a gift from Dr. Mach, Luzanne, Switzerland). A precipitate formed that indicated the presence of IgA. The concentrated material was used to antigenize rabbits for the production of anti-IgA. Two rabbits were antigenized with 0.5 ml. S-IgA (5 mgs%) and Freund's complete adjuvant given subcutaneously into 4 sites. The antigen was administered 4 times at 10 day intervals. The resulting rabbit anti-SIgA was tested for specificity by reacting the antiserum against purified bovine immunoglobulins using double micro-diffusion as described by Ouchterlony (1972).

A reaction of identity was detected between rabbit anti-SIgA and Mach's S-IgA antiserum and a single antigen in colostral whey. The antisera was treated further to ensure monospecificity for the heavy chain determinant, of S-IgA by passing through Sepharose 4-B columns containing CnBr bound IgM or IgG<sub>1</sub>. The purified antiserum gave a single band when reacted against colostrum of serum electrophoresed

in agar. The absorbed antiserum was again tested for specificity and reactivity by double diffusion experiments by comparing the precipitating activity against colostral proteins and serum proteins with the monospecific antiserum supplied by Mach.

A considerable effort was made to isolate secretory IgA from colostrum. Unfortunately, the concentration of IgG<sub>1</sub> is about 20 times the concentration of S-IgA in whey, hence, it was extremely difficult to separate the secretory immunoglobulins from the IgG's. Colostrum was treated with renin and calcium chloride as previously described in Materials and Methods and the whey was concentrated by pressure dialysis. The concentrated whey was passed through Sephadex G-200 after it was dialyzed against Tris-HCl buffer (0.03 Molar, pH 7.8). The whey was eluted with Tris buffer and collected in 3 ml. fractions. The elution data were plotted on semi-log paper, and the peaks were defined. The tubes comprising the leading edge of the first peak were pooled and concentrated to 10 ml. and again passed through G-200. The first portion of the leading peak was pooled and concentrated. The concentrated material was dialyzed against Tris-HCl buffer, 0.005 Molar, pH 8.0. The dialysate was layered on a DEAE column preconditioned with Tris-HCl buffer, 0.005 Molar pH 8.0. Two hundred ml. of this buffer was passed through the column before an elution gradient was started. The gradient was formed with appropriate mixing of

Tris-HCl buffer, 0.3 M, pH 5.0 to the Tris-HCl buffer of 0.005 Molar, pH 8.0. Proteins reacting with anti-SIgA and IgG<sub>1</sub> were found in the final peak in the elution profile. Repeated passage of the pooled material from the last peak did not resolve the two proteins. At one time during the separation procedures, the final peak eluates were concentrated and passed through a Sephadex G-200 column in an attempt to separate IgG<sub>1</sub> and S-IgA on the basis of molecular size. Separation of the two immunoglobulins did not occur. Continued passage through DEAE sephadex did not resolve the contamination problem.

Utilizing the purified antisera prepared against IgG<sub>1</sub>, IgG<sub>2</sub>, IgM and IgA in addition to the anti-SIgA supplied by Mach, it was possible to determine, by single radial-immunodiffusion quantitation procedures, that the final preparation of S-IgA from colostrum was 90% S-IgA and 10% IgG<sub>1</sub>. The total protein concentration in the solution was ascertained by absorbance measurements at 278 nanometers. After making a correction for the amount of IgG<sub>1</sub> contaminant, the solution was used as a reference standard in quantitative procedures. It was apparent that saliva was the better source than colostrum for isolating bovine secretory-IgA.

IgM was isolated from bovine serum. The serum was concentrated 2 fold by pressure dialysis and 3 ml. of the concentrate were applied to a 0.5 cm. x 8 cm. slit in a 18 x 45 cm. pevikon block. The

serum was electrophoresed at 400 volts for 24 hours in the cold. Sections 1 cm. x 8 cm. were cut from the block and placed in plastic vials. The bottoms of the vials had small holes drilled at regular intervals to allow passage of liquids. A filter paper disc (Whatman #4) was fitted to the bottom of the vial to retain the pevikon. The vials were set into large centrifuge tubes previously flattened along the rim to keep vials from descending the tube during high speed centrifugation.

The liquid expressed from the pevikon, using this system, contained the buffer solution (solvent system) and protein. Each fraction (eluate) was assayed for protein by absorbance spectrophotometry. A plot of the optical densities of the eluates vs. the pevikon fraction number indicated the areas in the block that had proteins present. Extracts contributing to the beta region of the electrophoretogram were pooled and concentrated. The concentrate was applied to Sephadex G-200 that had been preconditioned with a Tris-HCl (0.03 M, pH 7.6) buffer system. Fractions from the void volume were pooled and concentrated and passaged again through G-200. After two separations on Sephadex, the pevikon beta fraction was purified to yield a single band, near the origin, in gel electrophoresis. This material was used to antigenize for production of anti-bovine IgM serum.

Immunoabsorbant Columns

The procedure for coupling immunoglobulins to Sepharose 4-B was essentially the method described by Cuatrecasas et al. (1968). One gram of CnBr (one ml.) activated Sepharose 4-B was combined with 20-25 O.D. units per ml. of immunoglobulin in a 50 ml. beaker. The suspension was mixed, at a very slow speed, with a Magnamix for 24 hours at refrigerator temperature.

A five ml. aliquot of the suspension was added to a 10 ml. syringe fitted with a paper filter disc at the bottom to retain the Sephadex beads. The material was allowed to pack by settling and then was rinsed with copious amounts of PBS. The amount of protein in the washes was monitored by absorbance spectrophotometry and washing was discontinued when no further protein was eluted in the wash.

The technique was very efficient for the binding of IgG<sub>1</sub> and IgG<sub>2</sub> immunoglobulins to Sepharose beads. Measurements of the protein concentration before and after addition to the column (include all washes) indicated that 80% of the IgG<sub>2</sub> and 85% of the IgG<sub>1</sub> were linked to the beads. IgM did not bind as efficiently as the 7-S immunoglobulins. A binding efficiency of 40% was determined for the 19-S protein.

Immunoglobulin Heavy Chain Specific  
Antiserum

Vials of lyophilized IgG<sub>1</sub> and IgG<sub>2</sub> bovine immunoglobulin were purchased from Miles Laboratories (Kankakee, Illinois). The vials were reconstituted with 5 ml. sterile pyrogen free distilled water to obtain a stock solution of 10 mg./ml. protein. The stock solution was diluted with sterile saline to a final concentration of 2 mg./ml. and mixed with an equal volume of Freund's complete adjuvant for injection. Rabbits were antigenized subcutaneously with 1 mg. of protein distributed to four sites. Antigenation was repeated four times at four day intervals. Purified bovine IgM and S-IgA were administered with complete Freund's adjuvant in the same manner.

Rabbits were bled by cardiac puncture, five days before and 10 days after the final injection. The blood specimens were allowed to clot at room temperature for one hour before the serum was expressed by centrifugation. Tubes were spun at 10,000 rpm (9750 G) in a Sorvall RSB-2 refrigerated centrifuge. The sera were frozen in a Revco chest freezer and maintained at - 70°C. until processed.

Double diffusion in agar confirmed the specificity of the antisera. Each antiserum was tested for reactivity against each of the four major bovine immunoglobulins. Antisera demonstrating cross reactions were absorbed with the appropriate antigens by immunosorption. Monospecificity was ascertained by immunoelectrophoresis

and double immunodiffusion techniques. One percent agarose in barbital buffer (0.075 M, pH 8.6) was used for the electrophoretic and immunodiffusion studies. Bovine serum was electrophoresed from a well equidistant between two parallel troughs cut into the agarose. Anti-bovine serum was added to one trough and purified (absorbed) . . . monospecific anti-immunoglobulin serum was added to the other trough. Specificity was apparent when the single band formed by the monospecific serum fused with the appropriate band formed by the anti-bovine serum.

#### Quantitation of Immunoglobulins

The procedure used to measure the concentrations of the individual immunoglobulins in serum and colostral whey was a modification of the Masseyeff-Ziszwiler technique (Wilson et al., 1972). Ten mm discs were cut from 1% agarose layered in Hyland Diffusion plates. Antiserum was added to the discs at a concentration predetermined by experimentation. The discs were kept in the diffusion trays and maintained in a humid chamber throughout the experiment. The concentration of antigen (immunoglobulin) was adjusted so that the zone of optimum proportion for the antigen migrating from the well would occur at the midpoint between the edge of the well and the edge of the disc. A very rapid technique to determine the appropriate concentration of antigen was to dilute the antiserum in agarose and entirely fill a

diffusion tray. Then ten equally spaced wells in the agarose were prepared, serial dilutions of the antigen were added, and the concentration that formed a precipitin ring with a 4 to 6 mm diameter was selected.

The stock standard is diluted serially to obtain a number of different concentrations to devise a standard curve. The concentration of the working standards of purified immunoglobulin solutions were calculated from optical densities measured at 278 nm. The extinction coefficients used in these experiments were 13.8 for IgG<sub>1</sub>, IgG<sub>2</sub>, IgA and 12.8 for IgM. The curves indicate that a linear relationship exists between the concentrations of antigen and the diameter of the precipitin rings. Furthermore, the concentration of the antigen is directly proportional to the diameter of the precipitin ring. Therefore, by extrapolating values from a standard curve, the antigen concentration of an unknown can be determined.

#### System for Measuring the Concentration of Specific Elutable Antibody

An assay was developed to measure the specific antibody activities of IgG<sub>1</sub>, IgG<sub>2</sub>, IgM and S-IgA present in the serum and whey of vaccinated and non-vaccinated dams as well as in the serum of their offspring.

Since E. coli strain B-44 is a heavily encapsulated organism and was presumed to mask somatic antigens, it was deemed necessary to remove the capsular material (K antigen) by heating. Several trials revealed that heating at 118°C. under 18 psi for three hours was required to remove the K antigen from B-44. This heat requirement indicated that A type K antigen is present in the B-44 strain.

During initial work to devise a method to quantitate specific antibodies against the O-somatic antigens and the K capsular antigens, it was noted that after absorbing whey with cells boiled for 1 hour, less antibody was present to subsequently bind formalinized cells. Therefore, the cells were autoclaved for 3 hours to effectively remove the A type K antigen.

The following results obtained from absorption-elution methods confirmed the presence of K capsular material on E. coli cells heated at 110°C for one hour. One ml. of a 1 hour boiled cell suspension (450 Klett Units) was added to 0.3 ml. colostrum whey. The mixture was incubated for 2 hours at 37 degrees and then put into the refrigerator for an additional 4 hours. The suspension was centrifuged, the supernatant material was retained in a separate tube, and the cells were washed one time with PBS. The wash material and the first supernatant were combined and absorbed with one ml. of a formalinized B-44 culture (450 Klett Units). The formalinized organisms were the source

of the K antigen. The mixture was incubated at 37 degrees for 2 hours followed by a 4 hour incubation in the refrigerator (5-10°C.). Subsequently, the bacterial cells were sedimented and then washed one time with one ml. of PBS. Antibodies were eluted from the O-somatic and K capsular antigens with one ml. of 0.1 molar solution of aminoacetic acid. The acidified cells were repeatedly drawn into a pasteur pipette and expelled against the bottom of the tube to maximize dissolution of agglutinated cells. The resuspended cells were then centrifuged at 3000 rpm in a GLC table top centrifuge for 10 minutes. The supernatant, containing eluted antibody was pipetted directly into 0.4 ml. of 0.3 molar Tris-HCl, pH 7.8. Eluted immunoglobulins in the buffered supernatant were quantitated by the single radial immunodiffusion technique.

#### Passive Antibody Assay - A Murine Model

Neonatal pigs, sheep, calves, rabbits and dogs have been used with varying degrees of success to measure the effect of bacterial cells or their products in ligated intestinal segments. Even though ligated segments are formed with extreme care to ensure pristine intraluminal conditions, a system resembling a natural state would be more desirable. Therefore, neonatal mice were chosen for study because they are inexpensive, easy to obtain and can be obtained in large numbers.

Breeding cages contained five Balb/c female mice and one Balb/c male. The animals were fed Purina mouse chow and water ad libitum. Just prior to parturition the pregnant dams were put into private cages and maintained separately throughout the experiment. At parturition, all litters were adjusted in size to a maximum of eight neonates per dam.

Different volumes of enterotoxigenic materials and a variety of concentrations of live bacterial cells were tested in 2 to 7 day old mice to determine an effective challenge dose. A 0.20 ml. volume of an 18 hour culture of B-44, adjusted to contain  $10^5$  to  $10^7$  organisms per ml., was most effective if administered per os. Enterotoxigenic substances contained in 0.1 ml. of the supernatant material from an 18-24 hour culture (Trypticase Broth, Difco) was satisfactory if administered per os. Neonates challenged at 4 to 6 days of age were more susceptible to challenge than were older mice.

Both the challenge material and passive antibody were administered per os with a blunted 25 gauge hypodermic needle attached to a tuberculin syringe. Care was taken to avoid puncturing the esophageal passage or from depositing the material into the trachea.

In protection experiments a 0.1 ml. aliquot of the buffered glycine eluate containing anti-K elutable antibodies was administered 4 hours prior to challenge. Body weights were measured at 12 hour

intervals over a three day period. Body weight change was expressed as a growth index which was calculated in the following manner:

$$\frac{BW (O) - BW (T)}{BW (O)} \times 100 = \text{Growth Index}$$

BW (O) is the body weight in grams at the time of injection

BW (T) is the body weight in grams at time T

## RESULTS

### Passive Immunization in Experimental Colibacillosis

#### A clinical evaluation.

A. Characteristics of Strain B-44. In the preliminary study of strain B-44, several of the basic properties were determined. The most important of these was the confirmation of strain B-44 as an enteropathogenic strain of E. coli. Viable cells, filtrates, and concentrated toxin preparations caused distention of ligated intestinal segments in rabbits and calves.

Strain B-44 had both rough and smooth colony types, and both forms caused distention of ligated intestinal segments. However, only the smooth form of strain B-44 was used in these experiments.

The viable cell count of strain B-44 grown in heart infusion broth at 37°C. for 24 hours with vigorous aeration was approximately  $3 \times 10^{10}$  cells per ml.

B. Reponse of calves to oral challenge with live E. coli B-44. All but one of the 14 calves in the control group developed a 3 to 4 plus diarrhea (Table 1). The calf that failed to develop greater than a 1 plus diarrhea had been challenged at or before he was 12 hours old. A calf scour index that was derived by determining the mean diarrhea score for each experimental group from the data in Table 1 is shown in Table 2. The control calves had a scour index of

Table 1. Clinical evaluation of calves after challenge.

| Vaccine<br>Group of<br>Dam | Cow-Calf<br>Number | Signs of neonatal calf<br>diarrhea              |                                    | Score<br>(see text) | Birth of<br>Calf after<br>2nd<br>Vaccination<br>(days) |
|----------------------------|--------------------|-------------------------------------------------|------------------------------------|---------------------|--------------------------------------------------------|
|                            |                    | Onset of diarrhea<br>after challenge<br>(hours) | Duration of<br>diarrhea<br>(hours) |                     |                                                        |
| Control                    | 440-202            | 21                                              | 36                                 | 1 +                 | 10                                                     |
|                            | 422-209            | 7                                               | 40                                 | 4 +                 | 15                                                     |
|                            | 419-210            | 5                                               | 31                                 | 4 +                 | 17                                                     |
|                            | 443-212            | 7                                               | 48                                 | 4 +                 | 17                                                     |
|                            | 408-215            | 7                                               | 36                                 | 4 +                 | 19                                                     |
|                            | 405-221            | 6                                               | 27                                 | 4 +                 | 23                                                     |
|                            | 430-222            | 6                                               | 29                                 | 4 +                 | 24                                                     |
|                            | 427-225            | 11                                              | 43                                 | 4 +                 | 26                                                     |
|                            | 441-227            | 8                                               | 40                                 | 4 +                 | 28                                                     |
|                            | 620-229            | 5                                               | 32                                 | 4 +                 | 30                                                     |
|                            | 413-230            | 4                                               | 15                                 | 4 +                 | 32                                                     |
|                            | 446-234            | 4                                               | 20                                 | 4 +                 | 36                                                     |
|                            | 628-236            | 8                                               | 48                                 | 3 +                 | 41                                                     |
|                            | 411-240            | 7                                               | 33                                 | 4 +                 | 63                                                     |
| Crude<br>Toxin             | 442-201            | 11                                              | 8                                  | 1 +                 | 8                                                      |
|                            | 417-204            | neg                                             | 0                                  | 0                   | 11                                                     |
|                            | 622-207            | 12                                              | 48                                 | 1 +                 | 12                                                     |
|                            | 928-216            | 6                                               | 12                                 | 1 +                 | 20                                                     |
|                            | 630-228            | 9                                               | 33                                 | 1 +                 | 30                                                     |
|                            | 421-233            | 4                                               | 13                                 | 4 +                 | 36                                                     |
|                            | 433-235            | 4                                               | 72                                 | 4 +                 | 39                                                     |
|                            | 961-237            | 6                                               | 27                                 | 4 +                 | 43                                                     |
|                            | 410-238            | 17                                              | 29                                 | 1 +                 | 50                                                     |
| Live<br>Bacteria           | 650-206            | 11                                              | 33                                 | 4 +                 | 12                                                     |
|                            | 426-208            | 13                                              | 48                                 | 4 +                 | 15                                                     |
|                            | 403-211            | 7                                               | 24                                 | 1 +                 | 17                                                     |
|                            | 450-214            | neg                                             | 0                                  | 0                   | 19                                                     |
|                            | 429-217            | 6                                               | 36                                 | 1 +                 | 21                                                     |
|                            | 649-218            | 5                                               | 15                                 | 1 +                 | 22                                                     |
|                            | 407-223            | neg                                             | 0                                  | 0                   | 24                                                     |
|                            | 937-224            | neg                                             | 0                                  | 0                   | 24                                                     |
|                            | 922-239            | 4                                               | 25                                 | 1 +                 | 51                                                     |

Table 1 (continued)

| Vaccine Group of dam | Cow-Calf Number | Signs of neonatal calf diarrhea           |                              | Score (see text) | Birth of calf after 2nd Vaccination (days) |
|----------------------|-----------------|-------------------------------------------|------------------------------|------------------|--------------------------------------------|
|                      |                 | Onset of diarrhea after challenge (hours) | Duration of diarrhea (hours) |                  |                                            |
| Killed               | 432-203         | 0                                         | 0                            | 0                | 10                                         |
| Bacteria             | 618-205         | 7                                         | 48                           | 1 +              | 12                                         |
|                      | 948-213         | Born dead (cause unknown)                 |                              |                  | 17                                         |
|                      | 943-219         | neg                                       | 0                            | 0                | 23                                         |
|                      | 921-220         | 13                                        | 12                           | 1 +              | 23                                         |
|                      | 444-226         | neg                                       | 0                            | 0                | 24                                         |
|                      | 404-231         | Born dead (cause unknown)                 |                              |                  | 33                                         |
|                      | 439-232         | neg                                       | 0                            | 0                | 33                                         |
|                      | 434-241         | neg                                       | 0                            | 0                | 66                                         |

Table 2. Calf scour Index

| Vaccine group of Dam | Control | Crude toxin | Live Bacteria | Killed Bacteria |
|----------------------|---------|-------------|---------------|-----------------|
| Scour Index*         | 3.7     | 1.9         | 1.3           | 0.3             |

\* The index represents an average of the degree of scours based on the clinical evaluation of calves in each group. The averages are taken from the semi-quantative data that appears in Table 1.

3.7 which is markedly higher than the indices calculated for the calves in the vaccine groups.

Three of the 9 calves born to dams vaccinated with crude toxin developed a 4 plus diarrhea while 2 of 9 calves born to dams vaccinated with live bacteria showed a 4 plus diarrhea. Two calves in the killed bacteria group were born dead through unknown causes, therefore, only seven calves in this group were challenged. All seven of these calves were protected since none of them developed more than a one plus diarrhea.

The calf scour index as reported in Table 2 indicates that the calves in the killed vaccine group had the lowest index, 0.3, followed by 1.3 and 1.9 for the calves in the live bacteria and crude toxin groups, respectively.

The onset of diarrhea varied between 4 and 21 hours after the calves were challenged and was essentially the same for both the control and vaccine groups. The duration varied from 12 to 72 hours at which time the calves either had expired or were removed from the experiment.

Clinically, all calves gaining a score of 4 plus diarrhea had profuse, watery diarrhea which appeared several hours before the initial onset of the accompanying signs of dehydration (sunken eyes). As dehydration progressed apathy and anorexia appeared, and then the calf

was unable to stand, body temperature decreased, and if untreated, the calf became prostrate and died. Bacteremia was not detected until the calves were moribund. At necropsy, gross pathologic changes were not seen.

C. Bacteriologic evaluation of fecal specimens. Fecal swabs collected from 19 of 39 calves at time of challenge did not produce growth on Tergital 7 agar with triphenyltetrazolium chloride (TTC). In fecal swabs collected from the remaining calves at challenge, a few enteric bacteria were isolated. Typical morphologic features indicated the colony was of the rough form. Only two calves were excreting significant numbers of smooth colony types at the time of challenge.

After calves were challenged to strain B-44, the population of E. coli increased, with the majority being smooth and mucoid and having colonial morphologic features identical to those of strain B-44. Smooth organisms resembling strain B-44 were initially isolated from fecal swabs of every calf between 5 and 24 hours after challenge. The appearance of the various colony types remained constant for 4 days of fecal swab collections. Usually, rough colony types emerged and became approximately equal in number to the smooth, mucoid form by the fourth day of collection.

Serologic studies.

A. O agglutinins of the dam. The titration of preimmune sera from all of the dams against the O-somatic antigens of E. coli B-44 yielded undetectable or negative O agglutinins at all time intervals. The titration of cow serum two weeks after a single antigenation revealed that neither dams in the control group nor in the crude toxin group had detectable O agglutinins in their serum (Table 3). Dams vaccinated with live bacteria (LB) had a mean titer of 20 compared to a mean titer of 80 in the sera of the dams antigenized with killed bacteria (KB). These values are not significantly ( $p .05$ ) different (Table 4). The mean titer in serum from the KB groups are, however, significantly higher than the control group.

Assay of sera obtained at the time of parturition, which varies from 8 to 66 days after the second antigenation, detected antibody activity in the sera of the CT treated dams but the titers ( $= 21$ ) were very low. A slight increase in titer was seen in the sera of dams in the KB group ( $= 110$ ) while a three fold increase ( $= 63$ ) was observed in the sera of dams antigenized twice with live bacteria.

Investigations conducted a year after these titrations were performed indicated that the procedure used to prepare the O antigens was not rigorous enough to eliminate all of the capsular material from the bacterial cells. Therefore, the O agglutination titers reported

Table 3. Cow serum titers two weeks after a single antigenation and at parturition.

| Experi-<br>mental<br>group <sup>(f)</sup> | Anti-O <sup>(a)</sup>           |                            | Anti-K <sup>(b)</sup>           |                            | PHA <sup>(c)</sup>              |                            |
|-------------------------------------------|---------------------------------|----------------------------|---------------------------------|----------------------------|---------------------------------|----------------------------|
|                                           | Two weeks<br>post-<br>injection | At (e)<br>partur-<br>ition | Two weeks<br>post-<br>injection | At (e)<br>partur-<br>ition | Two weeks<br>post-<br>injection | At (e)<br>Partur-<br>ition |
| C                                         | <10 <sup>d</sup>                | <10 <sup>d</sup>           | <10 <sup>d</sup>                | <10 <sup>d</sup>           | 930 <sup>d</sup>                | 1274 <sup>d</sup>          |
| CT                                        | <10                             | 21                         | <10                             | <10                        | 2416                            | 5440                       |
| LB                                        | 20                              | 63                         | 10                              | 14                         | 1719                            | 6948                       |
| KB                                        | 80                              | 110                        | 10                              | 14                         | 2321                            | 6656                       |

a - Reciprocal of the mean titer of agglutinins to E. coli B-44 boiled cells.

b - Reciprocal of the mean titer of agglutinins to formalined E. coli B-44 cells.

c - Reciprocal of the mean titer of agglutinins to E. coli B-44 LPS-sensitized SRBC.

d - Serum heated at 56 C. for 30 minutes.

e - Parturition date varied from 8 to 66 days from the time of the second antigenation.

f - Dams vaccinated with placebo C; Crude toxin - CT; Live bacteria - LB; Killed bacteria - KB.

Table 4. Summary of statistical analysis\* of results in Table 3.

| Data analyzed           | Significance between groups** |    |           |    |
|-------------------------|-------------------------------|----|-----------|----|
| Anti-O (2 weeks P.I.)   | C                             | CT | LB        | KB |
| Anti-O (At parturition) | C                             | CT | <u>LB</u> | KB |
| Anti-K (2 weeks P.I.)   | C                             | CT | LB        | KB |
| Anti-K (At parturition) | C                             | CT | LB        | KB |
| PHA (At 2 weeks)        | C                             | CT | LB        | KB |
| PHA (At parturition)    | C                             | CT | LB        | KB |

\* Test of comparison among means by least square difference.

\*\* Means not underlined by the same line are different from each other (P<0.05).

Table 5. Calf serum titers at birth and at four days of age.

| Experi-<br>mental<br>group <sup>(f)</sup> | Anti-O <sup>(a)</sup> |                           | Anti-K <sup>(b)</sup> |                           | PHA <sup>(c)</sup> |                           |
|-------------------------------------------|-----------------------|---------------------------|-----------------------|---------------------------|--------------------|---------------------------|
|                                           | At<br>birth           | At four<br>days of<br>age | At<br>birth           | At four<br>days of<br>age | At<br>birth        | At four<br>days of<br>age |
| C                                         | <10 <sup>d</sup>      | <10 <sup>d</sup>          | <10 <sup>d</sup>      | <10 <sup>d</sup>          | <40 <sup>d</sup>   | 24 <sup>d</sup>           |
| CT                                        | <10                   | 56                        | 12                    | 60                        | 40                 | 214                       |
| LB                                        | <10                   | 90                        | <10                   | 240                       | <10                | 1920                      |
| KB                                        | <10                   | 97                        | <10                   | 121                       | <10                | 1496                      |

(a), (b), (c), d, (f) - Same as Table 3.

Table 6. Summary of statistical analysis\* of results in Table 5.

| Data analyzed                | Significance between groups** (P<.05) |    |    |    |
|------------------------------|---------------------------------------|----|----|----|
| Anti-O (At birth)            | C                                     | CT | LB | KB |
| Anti-O (At four days of age) | C                                     | CT | LB | KB |
| Anti-K (At birth)            | C                                     | CT | LB | KB |
| Anti-K (At four days of age) | C                                     | CT | LB | KB |
| PHA (At birth)               | C                                     | CT | LB | KB |
| PHA (At four days of age)    | C                                     | CT | LB | KB |

\*, \*\* - Same as Table 3.

include a minor contribution by anti-K antibodies that may be present in the specimen.

Colostrum whey from immune and non-immune dams was titrated both before and after heating at 56 °C. for 30 minutes. A very low titer of anti-O activity was detected in the unheated whey from the control dams (Table 7). It is interesting to note that before heating the whey, mean titers of 276, 134 and 77 were measured in the whey from dams antigenized twice with KB, LB and CT, respectively. These titers are significantly different at the 0.05 level (Table 8). After the whey was heat treated, the titers dropped by a factor of 65% in CT group, 32% in the KB group and 52% in the LB group. All of the titers, however, were still significantly higher than the mean whey titers of the non-immunized dams. It is important to note that the titer in the KB group remained significantly ( $p < 0.05$ ) higher than the titer in the other two vaccine groups. Thus, it appears that over 50% of the O agglutinin activity in the whey from dams antigenized with either CT or LB was related to a heat-labile immunoglobulin. On the other hand, only about 30% of the activity in whey from the dams antigenized with KB was sensitive to the action of heat.

Agglutinating activity was not detectable in whey from milk obtained from dams 3 months post-partum, in the control, crude toxin or live bacteria vaccine groups (Table 9).

Table 7. Colostral whey titers.

| Experi-<br>mental<br>group <sup>(f)</sup> | Anti-O <sup>(a)</sup> |                       | Anti-K <sup>(b)</sup> |                       | PHA <sup>(c)</sup>    |
|-------------------------------------------|-----------------------|-----------------------|-----------------------|-----------------------|-----------------------|
|                                           | Unheated              | Heated <sup>(d)</sup> | Unheated              | Heated <sup>(d)</sup> | Heated <sup>(d)</sup> |
| C                                         | 19                    | 10                    | <10                   | <10                   | 2,720                 |
| CT                                        | 77                    | 27                    | 14                    | 10                    | 12,581                |
| LB                                        | 134                   | 38                    | 39                    | 16                    | 21,809                |
| KB                                        | 276                   | 190                   | 69                    | 54                    | 37,050                |

(a), (b), (c), (d), (f) - Same as Table 3.

Note: The animals in Table 7 and Table 3 are not the same animals.  
Therefore, no attempt should be made to correlate the data.

Table 8. Summary of statistical analysis\* of results in Table 7.

| Data analyzed                            | Significance between groups** |           |           |    |
|------------------------------------------|-------------------------------|-----------|-----------|----|
| Anti-O (Unheated)                        | C                             | <u>CT</u> | <u>LB</u> | KB |
| Anti-O (Heated) <sup>d</sup>             | C                             | <u>CT</u> | <u>LB</u> | KB |
| Anti-K (Unheated)                        | C                             | <u>CT</u> | <u>LB</u> | KB |
| Anti-K (Heated) <sup>d</sup>             | C                             | CT        | LB        | KB |
| PHA <sup>(c)</sup> (Heated) <sup>d</sup> | C                             | <u>CT</u> | <u>LB</u> | KB |

\*, \*\* - Same as Table 3.

Table 9. Milk whey titers 3-4 months post-partum.

| Vaccine group <sup>(f)</sup> | Anti-O <sup>(a)</sup> | Anti-K <sup>(b)</sup> | PHA <sup>(c)</sup> |
|------------------------------|-----------------------|-----------------------|--------------------|
| C                            | <10 <sup>(d)</sup>    | <10 <sup>(d)</sup>    | 22 <sup>(d)</sup>  |
| CT                           | <10                   | <10                   | 80                 |
| LB                           | <10                   | <10                   | 60                 |
| KB                           | <10                   | <10                   | 80                 |

(a), (b), (c), (d), (f) - Same as Table 3.

B. O agglutinins of the calf. The O agglutinin titers were estimated on serum harvested from calves prior to colostrum feeding and 4 days post-parturition. The results of these titrations are found in Table 5. The raw data, not shown in the table, indicated that antibody activity was detectable in the serum of some of the neonatal calves. Less than three calves in each of the antigenized groups demonstrated titers, however, none of the titers exceeded 40. The data presented in Table 5 represents the mean titer for each group, which is less than 10 since so few of the neonates had antibody activity and the titers were extremely low. Reporting the mean titers as less than 1:10 is consistent with the procedure for all of the agglutination results.

The data show a significant (Tables 5 and 6) increase in the antibody titers in the sera of 4 day old calves born to KB, LB and CT

vaccinated dams as contrasted to the precolostral serum. Higher antibody titers against O-somatic antigens were found in the KB and LB groups than in calves in the CT group. It is important to note that none of the control calves had acquired detectable serum antibody that would react against the test organism by four days of age.

C. K agglutinins of the dam. Cow serum obtained before antigenation and at two weeks later were tested for antibody activity against the encapsulated E. coli B-44. None of the sera agglutinated a formalinized preparation of the bacteria before antigen was administered, nor at two weeks after the first vaccination was given (Table 3). At parturition, which varied from 8 to 66 days from the second vaccination, no antibody activity was detectable in the serum of dams in the control group, nor in the sera of dams antigenized with crude toxin. A very weak humoral response to the capsular antigens was detected only in the sera of dams that had been immunized twice with killed or live bacteria.

Titration was performed on whey before and after heating at 56°C. for 30 minutes. Neither the heated nor the unheated whey obtained from control dams had detectable antibody activity against the K antigens. An insignificant mean titer of 14 was measured in the unheated whey from dams in the CT group. The antibody contributing to this activity was inactivated by heat. The mean titers of the KB

group and the LB group of 69 and 39, respectively, are significantly (0.01) different (Table 3) and are also significantly higher than the control titers.

Heating the wheys from the KB group decreased the antibody activity by 22% while decreasing the activity in the LB group by 51%. Antigenation with live bacteria apparently influenced a greater production of heat-labile immunoglobulins than antigenation with the killed bacteria vaccine.

None of the milk specimens tested had detectable agglutinating antibody (Table 8) 90 to 120 days after calving.

D. K agglutinins of the calf. None of the serum specimens obtained from precolostral calves had detectable agglutinating activity to the K antigens of E. coli B-44 (Table 5). Furthermore, at four days of age none of the calves in the control group had detectable antibody activity against the capsular antigen(s). On the other hand, considerable antibody activity was acquired from colostrum by the calves in the CT, LB and KB groups. Calves in the KB group had a mean titer of 240 compared to titers of 120 and 60 in sera of calves in the KB and CT groups, respectively. The difference observed among groups are not statistically significant (0.05) (Table 5).

E. PHA activity of the dam. Antibody activity against the LPS moiety of B-44 was not assayed for in cow serum prior to antigenation. Two weeks after a single antigenation and again at parturition the cows were tested for serum PHA activity. The results are given in Table 3.

Following a single antigenation, the serum PHA activities detected in the vaccine groups were not significantly different (Table 4). However, the titers were significantly different at parturition which was 8 to 66 days after the second vaccination. The serum titers showed a three fold increase in all of the vaccine groups. The serum titers in the control group did not show an appreciable change.

A noticeable difference among the vaccine groups is seen in the colostral PHA whey titers (Table 7). The highest mean titer, detected in the KB group, has a mean of 37,000 which compares with a mean titer of 12,500 in the CT group and 21,000 in the LB group. The difference among these groups is not significant at the 0.05 level (Table 8). However, the results are significantly different between the vaccine groups and the controls. A mean titer of 2720 in the control group is 2 times the value noted for the PHA activity in the serum of the control dams (Table 3), whereas contrasting mean whey titers to their respective serum titers in the vaccine groups the vaccinated dams, at parturition, demonstrated 2 to 6 times more anti-LPS activity in the whey than in serum.

Relative difference between the vaccine groups (doubling of the PHA titers where  $CT < LB < KB$ ) is also seen in the results of the O antibody titers measured in unheated whey (Table 7).

#### Immunological studies.

A. Quantitative assays of immunoglobulins in the dam. Cow sera were assayed for immunoglobulin concentrations at the time of parturition, due to the variation of levels of immunoglobulins associated with calving (Butler et al. (1972)). Blood specimens from 2 cows in each group were analyzed by single radial diffusion techniques to determine the concentrations of serum IgG<sub>1</sub>, IgG<sub>2</sub>, IgA and IgM. The results of these studies are shown in Table 10. The number of samples tested was too small to apply a statistical evaluation, therefore, the differences that were noted were viewed on a relative basis.

It can be seen from these data that the serum levels of IgG<sub>1</sub> were not markedly different between the control and vaccine groups. These findings would indicate that none of the vaccine preparations influenced, in a detectable fashion, the total concentration of IgG<sub>1</sub> in the serum of the antigenized dams. On the other hand, the values obtained for serum IgG<sub>2</sub> showed marked variation between control and vaccinated dams as well as among the different vaccine groups. The mean concentration of IgG<sub>2</sub> in sera of the CT dams was nearly three times greater than control values and two times as high as levels in

the sera of KB dams. The sera from LB dams were not assayed for IgG<sub>2</sub>.

Table 10. Total immunoglobulin levels in the serum of control and test cows at parturition.

| Vaccine<br>group <sup>(f)</sup> | IgG <sub>1</sub><br>(mgs%)** | IgG <sub>2</sub><br>(mgs%) | IgA<br>(mgs%) | IgM<br>(mgs%) |
|---------------------------------|------------------------------|----------------------------|---------------|---------------|
| C (2)*                          | 1846                         | 706                        | 8             | 148           |
| CT(2)                           | 1437                         | 2008                       | 5             | 629           |
| LB(2)                           | 1482                         | nd                         | 13            | 457           |
| KB(2)                           | 1575                         | 1041                       | 8             | 644           |

mgs%\*\* - Concentration of IgG<sub>1</sub>, IgG<sub>2</sub>, IgA, or IgM reported as milligram per 100 ml. fluid.

( )\* - Number of animals tested.

(f) - Same as Table 3.

n.d. - Not done.

It was of interest to find that the serum levels of IgA, ranging from 5 to 13 mgs%, were highest in the sera of dams antigenized with live bacteria (LB). Serum IgA in the LB dams was nearly 3 times greater than serum levels detected in the CT groups. The concentration of IgA in the serum of control dams and KB immune dams was of the same magnitude but about 25% less than the levels found in the LB immune dams.

There was a distinct difference in the mean serum levels of IgM in control dams when contrasted to the values measured in sera of

dams in the vaccine groups. Mean serum levels, 629 vs. 644 mgs%, were not noticeably different between the CT and KB groups, respectively, whereas the LB dams had slightly lower values of IgM in their serum. These data would suggest that subcutaneous and intramammary antigenation had a greater influence on IgG<sub>2</sub>, IgM and serum IgA levels (Table 10) than on serum concentrations of IgG<sub>1</sub>.

As already stated in the Introduction of this paper, intramammary instillation of antigenic material has been shown to affect the levels of immunoglobulins in lacteal secretions. It had also been noted that selective concentration of immunoglobulins can occur in the mammary glands of the bovine. Therefore, a random selection of a number of whey specimens acquired from each vaccine group were assayed for IgG<sub>1</sub>, IgG<sub>2</sub>, S-IgA and IgM. The results of these studies are shown in Table 11.

Table 11. Total immunoglobulin levels in whey of control and test cows.

| Vaccine group (f) | IgG <sub>1</sub> (mgs%) ** | IgG <sub>2</sub> (mgs%) | S-IgA (mgs%) | IgM (mgs%) |
|-------------------|----------------------------|-------------------------|--------------|------------|
| C (6)*            | 4176 (3147)†               | 401 (405)               | 363 (336)    | 322 (329)  |
| CT(8)             | 1749 ( 990)                | 415 (434)               | 135 ( 26)    | 280 (240)  |
| LB(9)             | 2190 (1384)                | 390 (110)               | 183 (102)    | 502 (251)  |
| KB(6)             | 2721 (1832)                | 401 (202)               | 207 (114)    | 495 (280)  |

(f) - Same as Table 3.

( )\* - Same as Table 10.

( )\*\*- Same as Table 9.

† - One standard deviation.

The elevated mean concentration of IgG<sub>1</sub> in the colostrum of the control dams was due primarily to an inordinately high level of 7-S immunoglobulin in the right front quarter of one of the control dams used in the current study. The value, 9200 mgs%, detected in this one dam could not be attributed to a concentration of whey even though the sample obtained was noted to be of a lesser volume than routinely acquired for these studies. The raw data from this quarter was of particular interest since the level of IgG<sub>2</sub> was not at all unlike the concentrations seen in any of the other control wheys, whereas the IgM values were 25% lower and the S-IgA levels were nearly two times higher than the other whey values in the control group. Clinically, the quarter did not appear to be infected nor did the colostrum show indications of a chronic or acute udder infection.

A good example of greater variation occurring among quarters within a cow than among cows within different groups is seen in the results of the studies of colostrum IgG<sub>2</sub>. The mean concentration of each group clusters nicely about a value of 400 mgs%, while the between quarter and between cow variance is notably high (one std. deviation, Table 11). On the other hand, the data for S-IgA levels in the colostrum of control and vaccine groups show considerable variation among groups and among cows within groups. Even though the mean values are highly variable among cows within the groups, a

difference is clearly seen between the CT and control values, the former being about one third lower than the latter. There is little difference between the S-IgA levels in colostrum of dams vaccinated with either the LB or KB antigens.

The values obtained for colostral IgM reflect the relative distribution of S-IgA levels. Among the vaccine groups the CT dams had the lowest values while the LB and KB dams, which have similar mean concentrations (approximately 500 mgs%), are clearly higher than the levels observed in the serum of both the CT and control dams.

These data would indicate that both the LB and KB vaccines had a greater influence than CT upon levels of both IgM and S-IgA in the colostrum of parturient dams.

#### B. Quantitative assays of immunoglobulins in the calf.

Sera of the neonatal calves, obtained soon after they were born and prior to intake of colostrum, did not have detectable levels of IgG<sub>1</sub>, IgG<sub>2</sub> or IgA (Table 12). Of particular interest, therefore, was the finding that neonatal calves did have detectable levels of IgM in their sera. Even though the levels are low, in comparison to adult serum levels, it is important to note that calves not yet exposed to the normal environment have 19-S immunoglobulin in their serum. Therefore, finding higher levels of this early immunoglobulin in

calves, in the KB and CT groups, would indicate that antigenation influenced the immunological status of the fetal calves.

Table 12. Immunoglobulin levels in calf serum at time of birth (precolostral).

| Vaccine group <sup>(f)</sup><br>of dams | Immunoglobulin concentration |                            |               |               |
|-----------------------------------------|------------------------------|----------------------------|---------------|---------------|
|                                         | IgG <sub>1</sub><br>(mgs%)** | IgG <sub>2</sub><br>(mgs%) | IgA<br>(mgs%) | IgM<br>(mgs%) |
| C (13)*                                 | 0                            | 0                          | 0             | 8.4           |
| CT( 4)                                  | 0                            | 0                          | 0             | 17            |
| LB( 5)                                  | 0                            | 0                          | 0             | 12            |
| KB( 2)                                  | 0                            | 0                          | 0             | 21            |

(f), ( )\*, (mgs%)\*\* - Same as Table 10.  
0 - None detected.

At four days of age, it is apparent that the neonatal calves had acquired a considerable quantity of IgG<sub>1</sub> from the colostrum of the dam (Table 13). The total concentration of the 7-S immunoglobulin, without the contribution of IgG<sub>2</sub>, was about one-third the levels measured in adult cow sera at parturition. It was interesting to find that IgG<sub>2</sub>, at least quantities detectable with the methods employed, had been passaged to neonatal sera by day 4. On the other hand, a considerable amount of IgA was detected in the sera of all four day calves studied. Calves born to the LB group of dams had the highest mean concentration of IgA while the calves born to the dams vaccinated

with KB had markedly lower values. The data in Table 13 would indicate that variable efficiency for the absorption of colostral IgM from the intestinal tract of neonates exists among the groups, or that a relationship between the concentrations of immunoglobulins in colostrum had an effect on the levels of IgM in the sera of four day old calves. In this regard, it is interesting to note the obviously low values of IgM found in the serum of calves born to KB dams. These calves also had lower IgG<sub>1</sub> and clearly the lowest values of serum IgA at four days of age. However, it is curious that this group of calves were earlier distinguished among the three groups because of noticeably higher levels of IgM (Table 12) at the time of birth.

Table 13. Immunoglobulin levels in the sera of calves at 4 days of age.

| Vaccine group <sup>(f)</sup><br>of dams | Immunoglobulin concentration  |                            |               |               |
|-----------------------------------------|-------------------------------|----------------------------|---------------|---------------|
|                                         | IgG <sub>1</sub><br>(mgs%) ** | IgG <sub>2</sub><br>(mgs%) | IgA<br>(mgs%) | IgM<br>(mgs%) |
| C (4) *                                 | 648                           | 0                          | 22            | 69            |
| CT(6)                                   | 949                           | 0                          | 18            | 80            |
| LB(8)                                   | 649                           | 0                          | 25            | 83            |
| KB(4)                                   | 583                           | 0                          | 10            | 29            |

(f), ( ) \*, ( ) \*\*, 0 - Same as Table 10.

Specific immunoglobulins with activity against O and K antigens of strain B-44. Results of quantitative assays for total immunoglobulins did not correlate well with the scour indices. The vaccination procedures did elicit an immune response that was influential in the calf protection observed in the clinical studies. Since the results of the agglutination studies were only indicative, quantitation of immunoglobulins with specific activity against the somatic and capsular antigens were performed on whey and serum.

The results of these investigations are shown in Tables 14 to 19 and in the Appendix Tables A-1 to A-4.

A. Antibodies of the IgG<sub>1</sub>, S-IgA and IgM class in the dam.

Twenty-eight colostrum specimens from dams in the control group were assayed for anti-O antibody residing in each of three immunoglobulin classes; IgG<sub>1</sub>, S-IgA and IgM. It was interesting to find that IgG<sub>1</sub> anti-O immunoglobulin was present in all of the whey specimens at high values (Table 14), whereas anti-O of the IgG<sub>1</sub> class was found in 91, 84 and 86% of the whey samples from CT, LB and KB dams, respectively (Table 19). The mean concentration of colostral IgG<sub>1</sub> in the vaccine groups were not markedly different. In fact, the mean values of these groups formed a tight cluster about a derived mean of 17 mgs%. The mean levels of anti-O of the IgG<sub>1</sub> immunoglobulin class was nearly three times greater in the control dams than in the vaccinated dams.

Table 14. Average concentration of specific colostrum antibody in whey from control dams.

| Cow Number  | O-a (1)                 |              |            | K-a (2)                 |              |            | Calf scour index* |
|-------------|-------------------------|--------------|------------|-------------------------|--------------|------------|-------------------|
|             | IgG <sub>1</sub> (mgs%) | S-IgA (mgs%) | IgM (mgs%) | IgG <sub>1</sub> (mgs%) | S-IgA (mgs%) | IgM (mgs%) |                   |
| 413 (3,4) * | 49.2                    | 2            | 15         | 6.8                     | 0            | 0          | 4                 |
| 405 (3,1)   | 39                      | 1.7          | 42.9       | 0                       | 0            | 0          | 4                 |
| 427 (2,2)   | 68                      | 3.9          | 14.5       | 17                      | 0            | 0          | 4                 |
| 408 (2,2)   | 42                      | 2.1          | 26         | 33                      | 2            | 0          | 4                 |
| 440 (1,2)   | 34                      | 2.3          | 3.4        | 38                      | 0.25         | 0          | 0                 |
| 620 (2,2)   | 58                      | 2.7          | 49.4       | 25                      | 0            | 0          | 4                 |
| 422 (0,2)   | nd                      | nd           | nd         | 2.5                     | 0            | 0          | 4                 |

(1) - O-a, antibodies eluted from somatic antigens of E. coli B-44 cells heated at 118°C., 18 psi, 3 hours.

(2) - K-a, antibodies eluted from capsular antigens of E. coli B-44 cells treated with sodium azide.

0 - None detected.

n.d. - Not done.

( ) \* - Number of quarters tested (# tested for O-a, # tested for K-a).

\* - Calf scour index - see Materials and Methods.

Table 15. Average concentration of specific colostrum antibodies in whey from dams antigenized with crude toxin.

| Cow Number | O-a (1)                 |              |            | K-a (2)                 |              |            | Calf scour index |
|------------|-------------------------|--------------|------------|-------------------------|--------------|------------|------------------|
|            | IgG <sub>1</sub> (mgs%) | S-IgA (mgs%) | IgM (mgs%) | IgG <sub>1</sub> (mgs%) | S-IgA (mgs%) | IgM (mgs%) |                  |
| 421 (4,4)  | 15                      | 5            | 12         | 0.6                     | 0.5          | 13         | 4                |
| 928 (4,4)  | 11                      | 2.7          | 15         | 3.5                     | 1            | 8.5        | 0                |
| 410 (3,3)  | 6.3                     | 2.6          | 15         | 0.66                    | 3.3          | 22         | 0                |
| 417 (2,3)  | 8                       | 4.5          | 28         | 0.3                     | 1.6          | 4.6        | 0                |
| 442 (3,2)  | 15                      | 1.2          | 8.6        | 0                       | 0            | 0          | 0                |
| 433 (1,3)  | 58                      | 1            | 40         | 17                      | 0            | 0          | 4                |
| 622 (1,4)  | 17                      | 3            | 18         | 8                       | 0            | 0          | 0                |
| 630 (3,3)  | 39                      | 2.6          | 45         | 22                      | 0.3          | 0          | 0                |
| 961 (2,2)  | 9                       | 2            | 28         | 8                       | 3            | 0          | 4                |

(1), (2), 0, n.d., ( ) \* - Same as Table 14.

Table 16. Average concentration of specific colostral antibody in whey from dams antigenized with live bacteria.

| Cow<br>Number | O-a (1)                    |                 |               | K-a (2)                    |                 |               | Calf scour<br>index |
|---------------|----------------------------|-----------------|---------------|----------------------------|-----------------|---------------|---------------------|
|               | IgG <sub>1</sub><br>(mgs%) | S-IgA<br>(mgs%) | IgM<br>(mgs%) | IgG <sub>1</sub><br>(mgs%) | S-IgA<br>(mgs%) | IgM<br>(mgs%) |                     |
| 922 (4,4)*    | 9.5                        | 2.6             | 3.7           | 7.8                        | 0               | 0             | 0                   |
| 650 (3,3)     | 5                          | 6               | 17            | 4                          | 0.3             | 0             | 4                   |
| 429 (4,3)     | 1.4                        | 4.3             | 37.2          | 4.7                        | 0               | 51            | 0                   |
| 407 (2,4)     | 0.1                        | 4               | 29            | 7.3                        | 1.3             | 4.5           | 0                   |
| 426 (3,3)     | 33                         | 6               | 26.3          | 2                          | 1.6             | 11            | 4                   |
| 649 (1,2)     | nd                         | 19              | 46            | 11.5                       | 6               | 63            | 0                   |
| 450 (0,2)     | nd                         | nd              | nd            | 15                         | 7.5             | 0             | 0                   |
| 937 (0,2)     | nd                         | nd              | nd            | 4                          | 6               | 63            | 0                   |
| 403 (3,2)     | 58.3                       | 4               | 24.6          | 1.5                        | 2               | 0             | 0                   |

(1), (2), 0, n.d., ( ) \* - Same as Table 14.

Table 17. Average concentration of specific colostral antibody in whey from dams antigenized with killed bacteria.

| Cow<br>Number | O-a (1)                    |                 |               | K-a (2)                    |                 |               | Calf scour<br>index |
|---------------|----------------------------|-----------------|---------------|----------------------------|-----------------|---------------|---------------------|
|               | IgG <sub>1</sub><br>(mgs%) | S-IgA<br>(mgs%) | IgM<br>(mgs%) | IgG <sub>1</sub><br>(mgs%) | S-IgA<br>(mgs%) | IgM<br>(mgs%) |                     |
| 618 (4,4)*    | 10.8                       | 4               | 16            | 4                          | 2.3             | 10.8          | 0                   |
| 921 (4,4)     | 11.8                       | 9.5             | 15.8          | 12                         | 7.5             | 12.8          | 0                   |
| 444 (2,3)     | 8.5                        | 3.5             | 14.5          | 9                          | 3.6             | 6.7           | 0                   |
| 439 (2,3)     | 34                         | 2.5             | 21.5          | 0                          | 1.3             | 15.5          | 0                   |
| 948 (0,2)     | nd                         | nd              | nd            | 0                          | 12              | 4.5           | Dead @<br>birth     |
| 404 (1,2)     | 0                          | 3               | 10            | 7                          | 3               | 0             | Dead @<br>birth     |
| 437 (2,3)     | 22.5                       | 3.5             | 19.5          | 10.6                       | 0               | 0             | 0                   |

(1), (2), 0, n.d., ( ) \* - Same as Table 14.

Table 18. Average concentration of specific colostral antibody in whey.

| Vaccine group (f) | No. of qtrs. tested | Anti-O <sup>(1)</sup>   |              |            | Anti-K <sup>(2)</sup>   |              |            | Calf scour index |
|-------------------|---------------------|-------------------------|--------------|------------|-------------------------|--------------|------------|------------------|
|                   |                     | IgG <sub>1</sub> (mgs%) | S-IgA (mgs%) | IgM (mgs%) | IgG <sub>1</sub> (mgs%) | S-IgA (mgs%) | IgM (mgs%) |                  |
| C                 | 20                  | 48* (19)**              | 2.3 (1.0)    | 28 (18)    | 17 (14 )                | 0.3 (0 )     | 0 ( 0 )    | 3.7              |
| CT                | 28                  | 19 (17)                 | 3 (1.6)      | 21 (15)    | 7 (13 )                 | 1 (0.8)      | 5 ( 8.3)   | 1.9              |
| LB                | 25                  | 18 (23)                 | 4 (3.7)      | 22 (13)    | 6 ( 6.7)                | 2 (1.4)      | 16 (22 )   | 1.3              |
| KB                | 21                  | 16 ( 8)                 | 5 (2.4)      | 17 ( 8)    | 7 ( 4.6)                | 4 (3.8)      | 8 (13 )    | 0.3              |

(1), (2) - Same as Table 15.

( )\*\* - One standard deviation.

\* - Average concentration of immunoglobulin in colostral whey, derived from data in Tables A-1 to A-4.

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Table 19. Percent of quarters with detectable immunoglobulins with specificity to the somatic or capsular antigens.

| Vaccine group (f) | No. of qtrs. tested | O-a <sup>(1)</sup> |       |      | K-a <sup>(2)</sup> |       |     | Calf scour index |
|-------------------|---------------------|--------------------|-------|------|--------------------|-------|-----|------------------|
|                   |                     | IgG <sub>1</sub>   | S-IgA | IgM  | IgG <sub>1</sub>   | S-IgA | IgM |                  |
| C                 | 20                  | 100%               | 100%  | 100% | 66%                | 13%   | 0%  | 3.7              |
| CT                | 28                  | 91%                | 95%   | 95%  | 53%                | 34%   | 29% | 1.9              |
| LB                | 25                  | 84%                | 100%  | 80%  | 76%                | 48%   | 32% | 1.3              |
| KB                | 21                  | 86%                | 93%   | 93%  | 75%                | 52%   | 33% | 0.3              |

(f), (1), (2) - Same as Table 15.

Table A-3 shows that there was considerably more variation among IgG<sub>1</sub> levels in LB dams than in either of the other two vaccine groups.

The mean values of colostral IgA in control dams was not markedly different (Table 14), whereas dams in the vaccine groups (Tables 15,16,17) have an imposing variance among udders and also between cows. Values from 2.6 to 19 mgs% are noted in the LB group in contrast to 1.7 to 3.9 in controls. It is apparent from these data that vaccinating dams with live bacteria yielded a considerably different response in the udders of the dams. Except for the data on one cow (# 921) in Table 17, the S-IgA values in the KB group were not markedly different.

It was of interest that S-IgA anti-O was found in all udders tested from control and LB dams, whereas slightly fewer, 95 and 93% of the quarters of the CT and KB dams, respectively, contained this immunoglobulin.

When the whey specimens from control dams were analyzed, it was found that 100% of the specimens had detectable levels of IgM anti-O. The mean value of 28 mgs% (Table 19) compares favorably with mean levels of immune dams. The marked variation among quarters (Table A-1) and between cows (Table 14) in the control group of dams was not seen in the LB group (Table A-3 & 16) nor in the KB group (Tables A-4 & 17).

Mean values of anti-O immunoglobulins, when compared between groups, did not indicate an influence by the type of vaccine used in the group. In fact, the values in the whey of the vaccinated dams were generally lower in contrast to levels in the controls (Table 19). The lowest frequency for IgM anti-O occurred within the quarters of the LB group.

The mean group levels of colostral IgM anti-O did not relate well to their respective scour indices.

B. O antibodies of the IgG<sub>1</sub>, S-IgA and IgM classes in the calf. Quantitative analysis of the serum of newborn calves failed to show any of the immunoglobulins with activity against the O or K antigens. Serum from four day old calves were assayed and very low values for one class of immunoglobulin with specific antibody activity was found. The results of these studies are shown in Table 20. Only four calves in each group were tested, however, the data indicates that all of the calves acquired about the same quantity of anti-O IgG<sub>1</sub> immunoglobulins, whereas no detectable IgA or IgM immunoglobulins were present. Extremely low values for IgG<sub>1</sub> anti-K were found in the sera of the four day old calves. There may be a correlation between the levels of IgG<sub>1</sub> anti-K and the scour indices. The concentrations are so low that it precludes making any judgement on the significance of their presence in the infant calves.

Table 20. Specific immunoglobulin levels in serum of 4 day old calves.

| Vaccine<br>group (f)<br>of<br>dam | O-a (1)                       |               |               | K-a (2)                    |               |               | Calf<br>scour<br>index |
|-----------------------------------|-------------------------------|---------------|---------------|----------------------------|---------------|---------------|------------------------|
|                                   | IgG <sub>1</sub><br>(mgs%) ** | IgA<br>(mgs%) | IgM<br>(mgs%) | IgG <sub>1</sub><br>(mgs%) | IgA<br>(mgs%) | IgM<br>(mgs%) |                        |
| C (4) *                           | 1.2                           | 0             | 0             | nd                         | nd            | nd            | 3.7                    |
| CT(4)                             | 1.1                           | 0             | 0             | 0.4                        | 0             | 0             | 1.9                    |
| LB(4)                             | 0.9                           | 0             | 0             | 0.2                        | 0             | 0             | 1.3                    |
| KB(4)                             | 2.1                           | 0             | 0             | 0.65                       | 0             | 0             | 0.3                    |

(f), (1), (2), ( )\*, ( )\*\*, n.d., 0 - Same as Table 14.

C. K antibodies of the IgG<sub>1</sub>, S-IgA and IgM classes in the dam. Colostral IgG<sub>1</sub> with anti-K activity was found in 66, 53, 76 and 75% of the specimens tested from the C, CT, LB and KB dams, respectively (Table 19). The mean level of IgG<sub>1</sub> in the controls was twice as high as the means measured in whey from the vaccinated dams. It was interesting to find that with an overall group frequency of 53%, at least one quarter of each udder tested in the CT group had detectable IgG<sub>1</sub> anti-K. With the exception of cows 439, 407 and 442, this observation holds for the data of the other vaccine groups as well (Tables A-3 and A-4). Considerable variation in the mean udder levels was seen in the CT group data (Table 16). Variance was obvious in the other groups but to a much lesser degree. The presence of IgG<sub>1</sub> anti-K did not correlate, in magnitude at least, to the scour indices.

The data in Table A-1 show that only 13% of the control dams had detectable levels of S-IgA anti-K. The levels reported as specific anti-K immunoglobulins are indications of the concentration of immunoglobulins in the whey that were absorbed to sodium azide treated E. coli B-44 and subsequently eluted with glycine. Therefore, these antibodies represent, in essence, elutable antibody obtained with the techniques described in Materials and Methods.

Of particular importance were the data indicating that control whey contained insignificant levels of S-IgA anti-K (Tables A-1 & 14). It can be seen from the data in Table 14 that only 2 dams in the control group had detectable levels of S-IgA, whereas 34, 48 and 52% of the quarters assayed among CT, LB and KB dams, respectively, contained detectable levels of S-IgA. The values reported in the tables were related to the scour indices of their respective groups (Table 2). It was further noted from the data in Tables A-2, A-3 and A-4 that 6 of 9, 8 of 9 and 6 of 7 dams in the CT, LB and KB groups, respectively, had detectable levels of S-IgA anti-K in at least one quarter of the udders tested. This compares to the finding that only 2 of 7 udders in the control group had detectable levels of S-IgA anti-K.

Of signal importance is the data in Table 14 that indicates that all of the whey specimens from control dams lacked detectable levels of IgM anti-K immunoglobulins. Furthermore, all of the calves

born in this group attained a 4 plus diarrhea condition as shown in Table 1. The obvious lack of this immunoglobulin is an evidence of its significance in protection, especially when correlated with the clinical data in Table 1. To be sure, exquisitely low levels of S-IgA in addition to the low frequency (Table 19) contribute significantly to the understanding of the role of at least two immunoglobulins in passive immunity of neonatal diarrhea.

The data in Table 15 indicates that 3 calves were born to dams lacking both S-IgA and IgM in the colostrum; one of these calves scoured, whereas the other two did not. Cow #442 lacked all three anti-K immunoglobulins, yet the calf failed to scour. This calf had been challenged at 22 hours of age, which had been shown early, after that calves were much less sensitive to challenge after 10 hours of age.

Both of the calves that scoured in the LB group (Table 16) had been challenged earlier than 10 hours, at 5 hours of age, which also had been found in the clinical tests to be a time when the calves were exquisitely sensitive to oral challenge. An early challenge of a magnitude used in these experiments could likely overwhelm the early stages of passive immunity in neonatal calves.

It can be seen in Table 17 that two calves scoured, although the colostrum contained anti-K immunoglobulin. Additionally, all

immunoglobulins with anti-O specificity were present, however, the degree of scouring, 1 plus, is considerably less than that observed in the majority of the calves in the other groups and is not considered to be significant.

#### Enterotoxin and Live Bacterial Challenge of Neonatal Mice

Since heat stable (ST) and heat-labile (LT) enterotoxins have been implicated in neonatal diarrhea of swine and bovine (Smith and Halls, 1967), the effect of heat upon trypticase broth cultures supernatants (TBCS) of E. coli B-44 was tested. It can be seen in Table 21 that TBCS of B-44 was effective in causing a loss of body weight in neonatal mice. When heated (TBCS-H) at 120°C. for one hour, the weight loss was more pronounced. TB-H included as a control did not have a noticeable effect when given per os to neonatal mice. Thus, it is apparent that substances present in TBCS of the test organism adversely affected the weight gain of neonatal mice. E. coli strain 711(P-155) carries an enterotoxin plasmid (ent) that codes for the production of enterotoxin that has been reported to cause fluid accumulation in the ligated intestinal loop system in neonatal porcine and young rabbits (Gyles, 1973). This organism was included in these experiments to test the effect of a porcine-derived, enterotoxin-producing strain of E. coli in the murine system. It

can be seen in the results in Table 21 that E. coli 711(P-155) TBCS did not produce a detectable effect in the neonatal mice. The TBCS was not tested in ligated loops of neonatal porcine for activity, hence it is not possible to state whether the toxin was present in the TBCS or if it were present but not active in the mouse system.

TBCS most likely contained endotoxin as a result of the growth and death of E. coli in an 18-24 hour culture. Therefore, a solution containing 100 ugs (presumed to be more than ordinarily found in a broth culture) of B-44 endotoxin was administered to neonatal mice to test its growth retarding capacity. It can be seen in Figure 1 that neither heated nor unheated endotoxin produced a measurable effect upon the weight gain of neonatal mice.

E. coli strain Bx-13, isolated from the feces of a normal, conventionally reared mouse, was used in the model to determine if an enteric coliform derived from "normal intestinal flora" of the host animal would produce enterotoxic substances in TB. In addition, a gram positive organism, Bacillus subtilis, was included in the experiment to rule out the effects of metabolic products derived from bacterial growth which could not be attributed to endotoxin. It is obvious from the data in Figure 2 that B. subtilis did not contribute enterotoxic material to the TBCS. There was a toxic effect with TBCS-H and TBCS of both strain Bx-13 and strain B-44

Table 21. The effect of supernatant material\*\* on the weight gain of mice.

| Hours post-inoculation | Gain index after treatment with: |      |       |           |            |
|------------------------|----------------------------------|------|-------|-----------|------------|
|                        | TSB                              | B-44 | ΔB-44 | 711 (155) | Δ711 (155) |
| 15-20 (4) *            | 5                                | 5    | 4     | 11        | 11         |
| 21-26                  | nd                               | nd   | nd    | nd        | nd         |
| 27-32 (4)              | 14                               | 14   | - 1   | 20        | 20         |
| 33-38                  | nd                               | nd   | nd    | nd        | nd         |
| 43-48 (4)              | 20                               | 25   | 1     | 32        | 30         |

TSB - Trypticase soy broth (sterile), heated in boiling bath 1 hr.

B-44 - Supernatant material from an 18 hr. culture of B-44.

ΔB-44 - " " " " " " " " . In addition, this material was heated in a boiling water bath 1 hr.

711(155) - Supernatant material from an 18 hr. culture of E. coli 711(155). This strain of E. coli carries the plasmid for production of enterotoxin known to cause dilation of neonatal pig intestinal loops.

Δ711(155) - Same as above, except the supernatant material was heated in a boiling water bath for one hour.

**Gain index** - As described in Materials and Methods.

n.d. - Not done.

\* - Number of mice in an experimental group.

\*\* - 0.2 ml. administered orally to 5 day old mice.

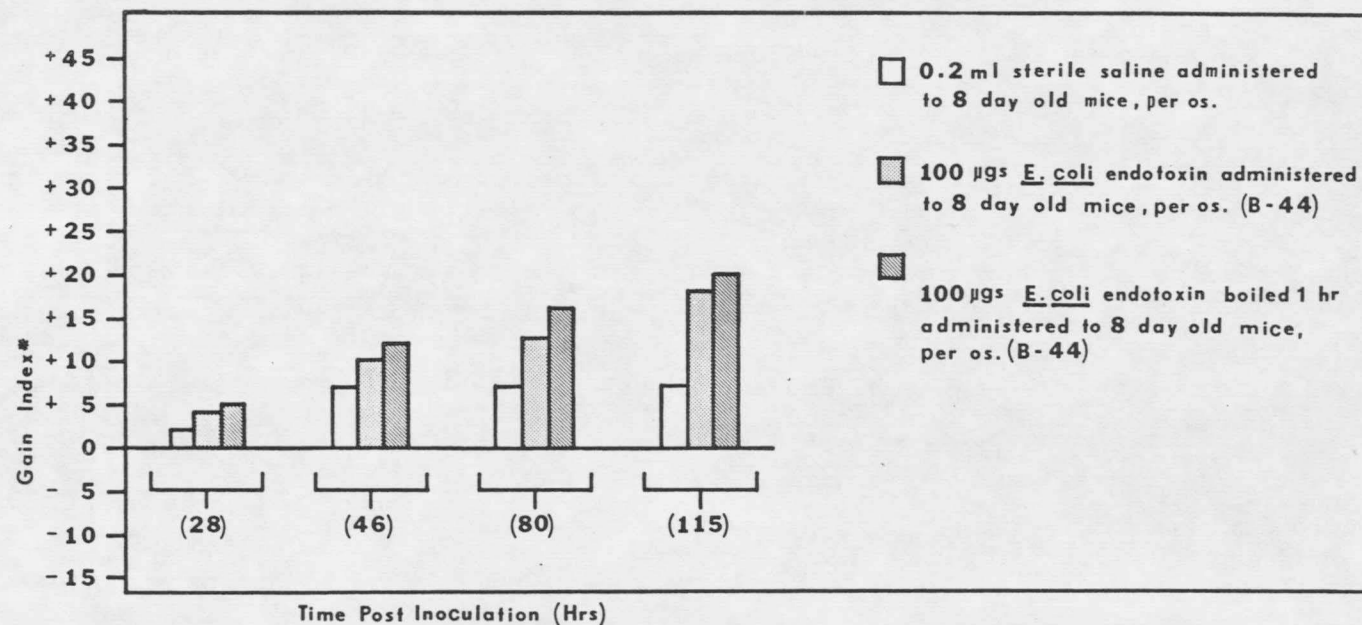


Figure 1. The effect of endotoxin on weight gain of mice  
\* see materials and methods

for the first 19 hours after challenge. However, this effect was not apparent 41 hours later in neonatal mice challenged with the TBCS of Bx-13. On the contrary, the most marked effect at 41 and 91 hours post-challenge was seen in the neonates that had been given B-44 TBCS-H per os.

It is known that heat-labile enterotoxins of certain E. coli strains can be neutralized with anti-ST:LT immune sera derived from animals antigenized with bacteria possessing the capability of producing ST:LT enterotoxin (Smith and Gyles, 1970). The ST enterotoxin of porcine strains is poorly antigenic and apparently poorly immunogenic. For this reason the initial studies designed to investigate the protective capacity of passive immunoglobulins involved a live challenge instead of the enterotoxin components of TBCS.

The data in Table 22 indicates that the most effective challenge doses for neonatal mice contain  $10^5$  to  $10^7$  B-44 organisms per milliliter. It further shows that the most appropriate interval, post-challenge, to measure the effect lies between 24 and 40 hours. Additional experiments (not recorded in the table) confirmed that 36 hours after oral challenge was the best time to measure the effect upon total body weight Gain Index (G.I.).

Since 7-day old mice were found to be partially resistant to oral challenge, 5- to 6-day old mice were used for subsequent investigations.

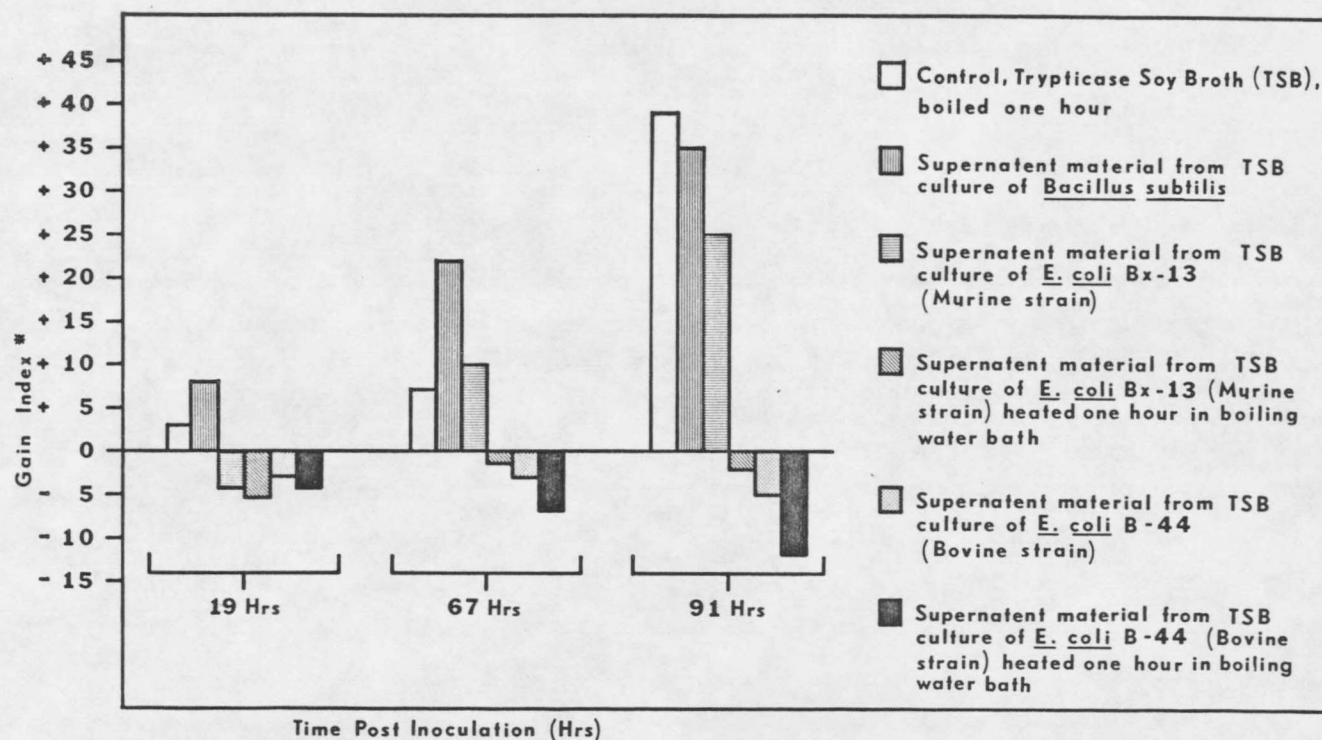


Figure 2. The effect of broth culture supernatant on weight gain of mice.\*\*

\* Gain Index, see materials and methods

\*\*0.2ml supernatant material administered to 7 day old mice per os

Table 22. The effect of different numbers of washed Escherichia coli B-44 on the weight gain of mice.

| Challenge dose**<br>No. bacteria/ml.<br>bacteria | Gain Index* |        |        |         |
|--------------------------------------------------|-------------|--------|--------|---------|
|                                                  | 24 hr.      | 48 hr. | 72 hr. | 120 hr. |
| $10^8$                                           | - 14        | - 10   | - 2    | + 5     |
| $10^6$                                           | - 17        | - 14   | - 10   | - 8     |
| $10^4$                                           | - 19        | - 15   | - 11   | - 9     |
| $10^2$                                           | - 16        | - 14   | 0      | + 5     |
| none                                             | - 12        | - 5    | + 2    | + 26    |

\* - Gain Index, as described in Materials and Methods.

\*\* - 4 seven day old mice challenged per os with each dose.

No deleterious effects were detected when glycine-buffer extraction reagents were used as controls in the experiments designed to test the effect of passive antibody in challenged mice (Table 23). If these animals were challenged 4 hours after glycine buffer treatment, however, a pronounced effect upon weight gain index was noted. A slight weight loss was noted in neonates shortly after manipulations only if a gavage tube was passed orally. These neonates recovered very rapidly from the trauma to make normal weight gains not unlike the control animals.

Animals were observed 4 to 5 days at which time only subtle differences were noticed among the controls and protected animals except in those cases where a very strong reaction was manifest very

Table 23. Effect of Passively Administered Colostral Antibody on the Weight Gain of Mice Challenged with Escherichia coli B-44.

| Antibody source<br>(quarter and dam<br>number) | Vaccine<br>Group | Treatment<br>(per os) | B-44 challenge<br>(per os) | Gain Index | Passive Protection<br>in |      |
|------------------------------------------------|------------------|-----------------------|----------------------------|------------|--------------------------|------|
|                                                |                  |                       |                            |            | Mice                     | Calf |
| Experiment #1                                  |                  |                       |                            |            |                          |      |
| RR 921                                         | CT               | anti-K**              | Yes                        | 10         | Yes                      | Yes  |
| RF 444                                         | KB               | anti-K                | Yes                        | 10         | Yes                      | Yes  |
| RR 437                                         | KB               | anti-K                | Yes                        | 9          | Yes                      | Yes  |
| RF 408                                         | C                | anti-K                | Yes                        | 6          | No                       | No   |
| none**                                         |                  | buffer                | Yes                        | 3          | --                       | --   |
| none*                                          |                  | buffer                | No                         | 8          | --                       | --   |
| Experiment #2                                  |                  |                       |                            |            |                          |      |
| LF 444                                         | KB               | anti-K                | Yes                        | 33         | Yes                      | Yes  |
| RF 921                                         | CT               | anti-K                | Yes                        | 12         | No                       | Yes  |
| LR 650                                         | LB               | anti-K                | Yes                        | 13         | No                       | No   |
| RF 427                                         | C                | anti-K                | Yes                        | 9          | No                       | No   |
| LR 417                                         | CT               | anti-K                | Yes                        | 26         | Yes                      | Yes  |
| none**                                         |                  | buffer                | Yes                        | 19         | --                       | --   |
| none*                                          |                  | buffer                | No                         | 26         | --                       | --   |

Table 23 (continued).

| Antibody source<br>(quarter and dam<br>number) | Vaccine<br>Group | Treatment<br>(per os) | B-44 challenge<br>(per os) | Gain Index | Passive Protection<br>in |      |
|------------------------------------------------|------------------|-----------------------|----------------------------|------------|--------------------------|------|
|                                                |                  |                       |                            |            | Mice                     | Calf |
| Experiment #3                                  |                  |                       |                            |            |                          |      |
| LR 423                                         | CT               | anti-K                | Yes                        | 14         | Yes                      | Yes  |
| RF 427                                         | C                | anti-K                | Yes                        | 2          | No                       | No   |
| LF 618                                         | KB               | anti-K                | Yes                        | 4          | No                       | Yes  |
| RF 413                                         | C                | anti-K                | Yes                        | 21         | Yes                      | No   |
| RF 620                                         | C                | anti-K                | Yes                        | 14         | Yes                      | No   |
| RR 948                                         | KB               | anti-K                | Yes                        | 20         | Yes                      | --   |
| LR 440                                         | C                | anti-K                | Yes                        | 16         | Yes                      | Yes  |
| RR 937                                         | LB               | anti-K                | Yes                        | 18         | Yes                      | Yes  |
| none                                           |                  | buffer                | Yes                        | 4          | --                       | --   |
| none                                           |                  | buffer                | No                         | 23         | --                       | --   |
| Experiment #4<br>(Wts. at 40 hours)            |                  |                       |                            |            |                          |      |
| LF 408                                         | C                | anti-K                | Yes                        | 21         | Yes                      | No   |
| LF 422                                         | C                | anti-K                | Yes                        | 22         | Yes                      | No   |
| RR 427                                         | C                | anti-K                | Yes                        | 31         | Yes                      | No   |
| RR 439                                         | KB               | anti-K                | Yes                        | 30         | Yes                      | Yes  |
| RF 444                                         | KB               | anti-K                | Yes                        | 21         | Yes                      | Yes  |
| RF 450                                         | LB               | anti-K                | Yes                        | 21         | Yes                      | Yes  |

Table 23 (continued).

| Antibody source<br>(quarter and dam<br>number) | Vaccine<br>Group | Treatment<br>(per os) | B-44 challenge<br>(per os) | Gain Index | Passive Protection<br>in |      |
|------------------------------------------------|------------------|-----------------------|----------------------------|------------|--------------------------|------|
|                                                |                  |                       |                            |            | Mice                     | Calf |
| LR 403                                         | LB               | anti-K                | Yes                        | 21         | Yes                      | Yes  |
| RR 417                                         | CT               | anti-K                | Yes                        | 19         | No                       | Yes  |
| none                                           |                  | buffer                | Yes                        | 19         | --                       | --   |
| none                                           |                  | buffer                | None                       | 26         | --                       | --   |

\*\*\* - anti-K treatment: K antigen, on sodium azide treated cells, was used to absorb whey. Antibody eluted from this antigen would be included in the material used to treat the neonatal mice.

Abbreviations - RR, Right Rear Quarter; RF, Right Front Quarter; LR, Left Rear Quarter; LF, Left Front Quarter; C, Control Group; CT, Crude Toxin; LB, Live Bacteria Group; KB, Killed Bacteria Group.

(\*\*) - Mouse Protection, challenged neonatal mice, administered passive antibody, had a higher gain Index than mice treated with glycine-buffer.

(\*\*) - Calf Protection, calves challenged with live Escherichia coli B-44 (per os) did not scour > 1+.

early in the test. In these cases the neonates were obviously runted and their gain index diverged precipitously.

It can be noted in Table 23 that only the anti-K antibody was tested for passive protection in the neonatal mouse system. The reason for using only anti-K antibody was based upon the findings (Table 18) that IgA and IgM immunoglobulins with anti-K activity were the only immunoglobulins not present in the whey of control cows, and further, that indications were very strong from the data collected in these studies that would implicate the signal importance of anti-K antibodies in passive immunity of neonates.

An apparent correlation between passive protection afforded neonatal mice by immune whey and the clinical evaluation of calf protection (degree of scours) was observed in 20 of 28 instances. The majority of the data that did not correlate were obtained with whey from control dams. Six of the 8 control specimens protected neonatal mice from challenge with B-44. Of particular interest is that in both cases, where control whey failed to protect both the neonatal mice and the calves, two quarters from each of their dams had been tested. One quarter in each case was protective, whereas the whey from the other quarter was not.

Whey from the left rear quarter of the control cow #440 protected neonatal mice, and furthermore, the calf born to this cow failed to scour after being challenged in the field experiments.

The results of tests using whey from vaccinated dams (Table 23) correlated in 12 of 15 instances. Of the three cases that did not agree, the calves were protected but the neonatal mice were not. Two of the three whey samples were derived from colostrum of dams antigenized with the crude toxin. The other source was whey from a dam vaccinated with killed bacteria.

## DISCUSSION

These studies, designed to evaluate the efficiency of three different vaccine preparations, indicated that subcutaneous and intramammary antigenation of preparturient dams afforded significant protection to the neonatal calves. Seventy-seven percent of the calves born to dams vaccinated with crude toxin (CT), live bacteria (LB) or killed bacteria (KB) were protected against an oral challenge with Escherichia coli B-44. On the contrary, only 2 of 14 calves born to dams in the control group failed to reach a 4 plus scour condition following oral challenge with the test organism.

The semi-quantitative index derived from the diarrhea score for each group of calves, showed that the KB vaccine was superior to the other three preparations in conferring passive immunity to neonatal calves. Next in the order of efficiency was the LB vaccine followed by the CT vaccine preparation. The CT preparation apparently contained enough somatic and capsular material to elicit a response sufficient to react with the challenge organisms and passively protect the neonatal animals.

It was noted, early in the experiments, that the time of challenge had a measurable effect on the degree of diarrhea induced by the B-44 strain. A challenge after 20 hours of age was clearly less detrimental to the calf than an oral challenge administered within the first 10 hours of life. Since the only calf in the control

group that had less than 3 plus diarrhea was also the calf that had been challenged beyond 12 hours of age, a relationship between age and susceptibility was suggested.

It is known that newborn calves absorb proteins more efficiently during the first 18-20 hours of life (Fey, 1971), and further, that strains of enteric organisms begin to establish the intestinal tract at 18 to 24 hours of age (Smith, 1965). It would follow then that transfer of bacterial enterotoxins as well as immunoglobulins, specific or of low avidity, would be influenced by the time related physiological mechanisms in the neonatal intestine. In addition, it is possible that endotoxin and enterotoxic substances are being released from dead and metabolically active bacteria, respectively, during the time period when the small intestine is exquisitely sensitive and vulnerable to molecular cytotoxic reaction. These factors quite likely contribute to field cases of neonatal diarrhea (scours) as well.

The pathogenicity of strain B-44 is proved by results in the present experiment and is corroborated by the results reported by Smith and Halls (1967) in which 3 calves challenged with  $10^{10}$  viable strain B-44 six to twenty hours after birth had severe diarrhea and dehydration. Of 13 control calves in the present experiment, 12 calves had 4 plus diarrhea. One control calf (236) responded atypically after challenge. This calf had severe diarrhea which was

initially followed by moderate dehydration and apathy. The dehydration and diarrhea ceased 48 hours after challenge. The calf was, however, in a weakened condition and probably did not nurse and died 5 days later.

The time of onset and duration of the clinical syndrome of the disease caused by strain B-44 (profuse diarrhea, severe dehydration, apathy, decrease in body temperature and morbidity; death) and the absence of bacteremia before the moribund state was reached, or any gross pathologic changes were seen, are typical of field cases of the disease. Bywater (1970) reported that physiological changes caused by strain B-44 enterotoxin included a net increase in secretion of fluid, sodium, bicarbonate and chloride from the blood into the small intestine of the calf. Similar physiologic changes in calves have been associated with naturally occurring neonatal diarrhea (McSherry and Grinyer, 1954; Dalton et al., 1965; Fisher, 1965).

Blood samples were obtained from dams at the beginning of this experiment, again 2 weeks after the second antigenation and finally at parturition. In addition, colostrum was collected from each quarter of each dam before the calf was allowed to suckle. Blood specimens were obtained from calves before they reached the age of 1 hour (frequently within minutes of birth) and again at four days of age. These samples were analyzed for activity against somatic and capsular

antigens using absorption and elution techniques described in this paper. The serologic and immunological investigations were performed to gain a better insight into the immunobiology of calf scours.

The results of these studies show that two weeks after a single antigenation a significant agglutinin response (neither anti-O nor anti-K) was not detectable in serum of dams. At parturition (8 to 66 days after the second antigenation), only anti-O serum titers in vaccine groups were significantly different from serum titers of the control dams. Anti-K activity still was not detected at parturition in the sera of the controls or the vaccine groups. These data agree with the findings of others that the O-agglutinating titers in sera of vaccinated dams are generally higher than are the titers detected against capsular antigens of the vaccine strain (Gay et al., 1964).

In the present studies, the KB, LB and CT vaccine, in that order, were effective in eliciting a response against the O-somatic antigens. No such ordering of vaccine efficacy was possible using anti-K titers. Therefore, since both anti-O and anti-K antibody activity are highly likely to be involved in passive immunity, an ordering of the effectiveness of KB, LB and CT vaccines from the results of serum activity of the dam clearly is not possible.

Since the importance of colostrum for the survival of the calf has been established (Fey, 1971; Ingram et al., 1953;

Aschaffenburg et al., 1949), an evaluation of the three vaccines for production of protective factors in colostrum would be an appropriate indication of vaccine efficiency. Since colostrum-derived antibody had been shown by many investigators to be a critical factor in passive immunity in neonatal calves (Briggs, 1951; Briggs et al., 1951; Wood, 1955), it would follow that an effective vaccine should elicit protective antibodies that will predominate in the dam's colostrum.

Anti-O activity in the whey of control and vaccinated dams was measured with E. coli B-44 heated cells (112°C for 2 hours). The antigens remaining after this heat treatment were reacted against both heated (56°C. 30 min.) and unheated whey obtained from individual quarters of the udder of each cow. In all of the groups, controls included, the titers detected in the unheated whey were distinctly higher than the corresponding titers in the heated specimens. The greatest response, measured as agglutinating activity, was noted in whey from dams antigenized with KB. The agglutinating titer in the LB group was one-half the titer detected in the KB group. Surprisingly, the mean agglutinin response made by the dams in the CT group was one-half the titer detected in the LB group. The control dams had very low agglutinin titers to E. coli strain B-44 somatic antigens which would likely be due to the low avidity, highly cross-reactive antibodies against the common antigen detected with the Enterobacteriaceae microorganisms.

It should be pointed out that all four immunoglobulin classes in the bovine are not equally effective in the agglutination reactions, hence, these agglutination titers most likely indicate activity of 7-S IgG<sub>1</sub> and 19-S IgM, IgG<sub>2</sub> and IgA may not contribute too substantially to this particular bacterial immunoassay.

One of the more interesting findings in the agglutination studies centered about the effect of heat upon the whey titers, LB and KB titers that were not significantly different ( $P > 0.05$ ) in unheated whey were found to differ significantly when measured in the heated whey specimens. Furthermore, the mean CT titers were no longer different ( $P > 0.05$ ) from the control titers after the whey samples were heated (whey samples were heated prior to titration). Curiously, the relationship observed between LB and CT titers, nor being significant ( $P > 0.05$ ), was not unlike the relationship observed in the heated or unheated titrations.

The effect of heat upon whey agglutinins (anti-O) was seen in the results of the anti-K titrations as well.

Anti-K titers in colostrum whey were also analyzed by the Least Square Difference statistical test. Titers of heated whey from dams antigenized with CT were not significantly higher than the titers in the control group. In addition, the mean anti-K titer in the LB group was significantly ( $P > 0.05$ ) lower than the mean titer in the KB

group. However, both the KB and LB groups had mean titers significantly higher (0.05) than the mean titers in control whey. These data indicate that the CT was not as effective in stimulating anti-K agglutinins as KB or LB. Moreover, the efficacy of the KB and LB vaccine preparations, as reflected by anti-O and anti-K titers, was appropriately reflected in the scouring index as well.

The mean anti-K titer in unheated whey from the CT dams, although low, was significantly higher than the mean titer of the control group; this was not observed with the heated whey. Additionally, in the presence of the heat-labile factor(s), titers of anti-K and anti-O were not significantly different between the KB and LB groups, which is contrary to the findings for heated whey. The effect of the factor upon the agglutinating activity in whey is apparent; the effect in vivo is unknown.

It is of interest that agglutination studies, using heat treated serum of the bovine, have been used in procedures for the diagnosis of brucellosis. Serum agglutinating activity in bovine serum that is decreased following heat treatment is generally observed in cows with early brucella infection and also in post-vaccination serum (Herbert, 1970).

Whether or not the heat-labile factor(s), present in whey of dams, represents an antibody to common enterobacterial antigen was not determined.

These studies would suggest that titers of anti-O and anti-K activities in heat-treated whey reflect differences in responses to the different vaccines. Clinical data, a scour index of 3.7 in control calves, and indices of 1.9, 1.3 and 0.3 in the CT, LB and KB calves, respectively, indicated that substantial protection was afforded calves in all vaccine groups. If agglutination titers measured in heated whey were used to predict the efficiency of the different vaccines, only the LB and KB vaccines would be expected to elicit protection. However, a prediction based upon the significant results of the agglutination studies of unheated whey would indicate that all three vaccine preparations would be effective. This latter prediction correlates more strongly with clinical results obtained in these studies, and suggests the complicity of a heat-labile immune factor in protection from scouring.

The PHA titers in serum of vaccinated cows, two weeks after a single antigenation and also at parturition, were significantly higher than titers observed in the sera of control cows. None of the mean titers in any of the vaccine groups, however, were significantly different. The titers increased in magnitude by a factor of two from the time of the second antigenation until parturition which occurred from 8 to 66 days after the last antigenation. The mean PHA activity in the wheys of the vaccine groups was significantly higher than the mean

titer in the control whey. Again, the differences in magnitude of the titers between the vaccine groups were not significant (0.05). Even though the mean PHA titers in whey are not significantly different in the vaccine groups, a relative difference was seen that is similar to differences noted in the data for anti-O titers of both heated and unheated whey. Furthermore, the relatedness of mean PHA and anti-O titers in whey specimens correlate well with the clinical findings.

Since endotoxin has not been shown to be a critical effector in E. coli enterotoxemias (Truszczyński and Ciocek, 1972; Smith and Halls, 1967; Gyles and Barnum, 1969), it is doubtful if the PHA agglutinating antibody functions in the protection against E. coli. It should be considered, however, that immunological or physiological activities contributing to death of enteric organisms coupled with a breakdown of the integrity of the intestinal mucous membrane (produced by enterotoxin) may require the interaction of endotoxin neutralizing antibodies for protection. It is apparent from these studies that anti-LPS agglutinating antibody is present in significant amounts in the serum and whey of the vaccinated dams and, like the anti-O agglutinins, deserve consideration as a significant factor in scouring.

Precolostral calves are usually agammaglobulinemic or have only traces of immunoglobulins in their serum (Husband et al., 1972; Merriman, 1971). Fetal calf circulation is isolated from maternal

circulation by an epithelialchorial placenta which effectively blocks the transfer of most large molecules of maternal origin (Brambell, 1970). Therefore, the presence of agglutinin activity in neonatal serum generally indicates colostral uptake and intestinal absorption of antibody proteins in the small intestine; the proteins pass through the epithelial cells and enters the lymphatics (Comline et al., 1951). Globulins may be detected in the thoracic duct one to two hours after introduction into the duodenum; very little if any globulin enters the portal circulation (Comline et al., 1951). Absorption of colostral antibodies is limited to the first 24 to 36 hours of life, with only traces of globulin being absorbed thereafter. It is not surprising then that at birth the calves in the present studies did not exhibit agglutinating activity against either the O or K antigens. Of particular interest is that anti-PHA activity was detected in precolostral calf serum in each of the groups, including the control calves. The titers ranged from a low of 30 to a high of 943 in the sera of calves born to dams antigenized with live bacteria. It is possible that in utero exposure to an LPS moiety would account for the antibody activity since immunocompetence has been demonstrated in another ungulate, the ovine (Silverstein et al., 1963) and more recently, in the fetal calf (Richardson and Conner, 1972; Conner, Richardson and Carter, 1973; Gay, 1971). Richardson and Conner showed that an antibody

response can be elicited in fetal lambs when brucella antigen is introduced into the amniotic fluid. It was noted by these investigators that the fetus synthesized high levels of anti-brucella agglutinins after it was stimulated late in gestation. However, in the ovine fetuses vaccinated at midgestation, the response was slow and with low levels of antibody. The response was adequate, however, to prime for secondary antigenic stimulation whether the latter was given in utero or at birth.

The recent report by Conner et al. (1973) indicated that prenatal vaccination (oral route) of bovine fetuses by depositing E. coli organisms (formalinized) in amniotic fluid elicited low titers of O agglutinins that could be detected in the sera of newborn calves. Although the agglutinin titers were low, protection against oral challenge with the live vaccine strain was afforded the neonate at birth.

Gay (1971), on the other hand, immunized four fetal calves intramuscularly with E. coli antigens with adjuvant, and one calf with E. coli antigens by depositing the antigens in the amniotic fluid. All calves responded to antigenation as determined by serum agglutination studies at birth. More importantly, however, the vaccinated calves were protected against oral challenge with live E. coli. Two imposing findings were seen in these data: (1) the vaccinated calves survived challenge with enterotoxic coliform organisms with serotypes

unlike the vaccine strain, and (2) greater cross reactivity to non-vaccine serotypes were noted in those calves that had been vaccinated in utero for the longest period of time.

Sera of four day old calves in this study yielded an increase in antibody activity in all of the calves in the vaccine groups while no additional antibody activity was seen in sera of the control calves. It is interesting that the mean anti-O titers were not significantly different between groups of immune calves since the anti-O colostrum titers of the dams in these groups were significantly different. A considerable increase in the anti-K activity was also noted in the sera of calves born to the control dams. The control dams consistently demonstrated a lack of anti-K activity in all serum and whey specimens.

The presence of antibody activity in the serum of the neonate serves a vital role until the immune system of the neonate becomes functional. In this regard, it is interesting that the reported half life of E. coli colostral antibodies in the calf has a mean value of 23 days (Comline et al., 1951). Therefore, once the calf acquires colostral antibody, it is available beyond the neonatal period of life.

Results of the quantitative measurements of immunoglobulins in whey show marked variation of the globulin levels between quarters and cows. Since the results were not normalized with the aid of a marker

in the whey, the effect of varying fluid secretion, in volume and composition, among individual quarters, cannot be interpreted. The marked variance observed among quarters in these studies, however, is not unprecedented. Butler et al. (1972) observed marked variation in immunoglobulin concentrations among quarters and cows. Their findings indicated the variance may have resulted from physiological factors other than dilution.

The mean concentrations of the four immunoglobulins in whey do not vary significantly among the vaccine groups. An unusually high IgG<sub>1</sub> value in one of the quarters of a control cow contributed to the difference observed among the IgG<sub>1</sub> results. It was noted at the time the colostrum samples were assayed that the volume of the specimen was less than the usual sample volumes. It was not ascertained whether the small volume was related to a decreased lactation or if the milker was unable to obtain the standard volume. If the former were the case, a concentration of the lacteal secretions may have been the contributing factor. The immunoglobulin data for this one quarter indicated that the IgG<sub>2</sub> level was normal for this group, whereas the IgM value was 25% higher and the S-IgA was nearly two times higher than the whey values in quarters of the control dams tested. Cross examination of the colostrum did not show any obvious signs of inflammation. However, the high levels of both IgG<sub>1</sub> and S-IgA in colostrum could easily result from an in-situ inflammatory reaction.

The mean concentrations of colostral IgG<sub>1</sub> in all groups (except the control) relate well to the values reported in the literature by Wilson et al. (1972), Husband et al. (1972) and Butler et al. (1972); the values are considerably less than the values reported by Mach and Pahud (1971) and Porter (1972). The cattle breeds used in the investigations mentioned above were Jersey, Ayrshire and Jersey and mixed breed, Holstein-Friesen, Simmental, and Ayrshire, respectively. Beside the differences frequently observed between different laboratories, it would appear that the breed of cattle used in the experiment may have an effect upon results of similar quantitative studies.

The IgG<sub>2</sub> concentrations did not appear to be influenced, even in a relative way, by the administration of different vaccines. Furthermore, with the exception of the levels reported for the Simmental cattle, the measured values for IgG<sub>2</sub> were notably similar to the values reported in the literature.

Colostral IgA values were not significantly different among the vaccine groups, although a relatively lower value is seen in the whey from dams antigenized with crude toxin. Interestingly, the variance among cows in this particular group is extremely low. Following intramammary antigenation, a pronounced effect upon the levels of secretory immunoglobulins would be expected. However, a significant

difference was not found among the total IgA levels in vaccinated and control dams. Recent work by Wilson et al. (1972) indicates that significant differences in the levels of S-IgA and IgG<sub>1</sub> in whey from E. coli antigenized and non-vaccinated quarters is not apparent until 3-4 days post-partum. At this time, and at weekly intervals thereafter, quantitative data indicated that S-IgA and IgG<sub>1</sub> remained significantly higher in the vaccinated quarters while the values in the non-vaccinated declined. The levels remained significantly higher until about the 28th day post-partum. Because the experimental design of the present project was basic to the development of a vaccine rather than to a scientific investigation of the immunobiology of neonatal diarrhea, the immunological studies cannot be conclusive. It is very likely that quantitative studies of whey specimens collected at prescribed intervals, post-partum, would agree with the results reported by Wilson et al. (1972).

The concentration of IgM was not significantly different among the vaccine groups and the controls. IgM was notably lower in the whey of the dams antigenized with CT. The high degree of variation observed with all of the immunoglobulin studies precludes analyzing the data for the effectiveness of different vaccine preparations based upon the levels of colostral immunoglobulins.

The four immunoglobulin classes were quantitated in sera of 2 cows each from the vaccine and the control groups. Only an

indication of the magnitude of the levels of the various immunoglobulins can be derived from this data since the number of observations in each group is small. Very little variance accompanied the quantitative data on levels of IgG<sub>1</sub> and IgM measured in serum of dams in the vaccine groups. IgG<sub>1</sub> levels were tightly clustered about a mean level that did not differ between vaccine and control groups. On the other hand, IgG<sub>2</sub> levels in the CT group were clearly higher than the control levels, and additionally, they were twice as high as values in the serum of dams in the KB group. No data was available for the IgG<sub>2</sub> concentration in serum from dams antigenized with LB vaccine.

These data indicate that IgA values were not affected by the vaccination procedures used in these studies. However, the IgM levels were 4 to 6 times greater in vaccinated dams than in the control dams. Unfortunately, the use of small numbers precludes significance testing of the variation among groups, however, the magnitude of the difference is apparent. Established values are not available for serum levels of IgG<sub>1</sub>, IgG<sub>2</sub>, IgA and IgM, hence, the values reported by Mach and Pahud (1971) will be used for comparisons. Mean values of IgG<sub>1</sub> were slightly higher in the serum of all dams than the values reported in the literature. No effect upon the serum levels of IgG<sub>1</sub> related to the antigenation regimes were noted in these studies. On the other hand, a marked increase in the IgG<sub>2</sub> levels is apparent in serum of dams

antigenized with CT. This increase is related to the use of adjuvant with the subcutaneous challenge, which maintains material at the injection site for a prolonged period, allowing the immune response to reach maturity.

Recent studies by Butler et al. (1972) indicate that IgG<sub>2</sub> comprises about 50% of the total serum IgG, which is in agreement with the reported values of Mach and Pahud (1971). In our studies, the IgG<sub>2</sub> contribution to the total concentration of IgG in serum of control dams was only 39%. Even though the total serum IgG<sub>2</sub> in both the vaccinated groups tested were markedly higher than control values, only in the CT dams was the relative concentration of IgG<sub>2</sub> demonstrable higher.

The increased levels of serum IgG<sub>2</sub> did not correlate with the IgG<sub>2</sub> levels in the colostrum. It has been clearly shown by Porter (1972), Murphy et al. (1964) and Dixon et al. (1961) that IgG<sub>1</sub> is selectively transferred from serum to mammary glands. The transfer of IgG<sub>2</sub> is much less efficient as evidenced by low concentrations of this immunoglobulin in the colostrum. Of particular interest, IgG<sub>2</sub> was observed in high concentration in the intestinal secretions of young calves (Porter, Noakes and Allen, 1972). Their studies showed that IgG<sub>2</sub> levels were higher than levels of IgG<sub>1</sub> in intestinal secretions from Thiry-Vella loops in 2 to 6 week old calves. In one of three

calves studied, IgG<sub>2</sub> was consistently the major immunoglobulin in the intestinal secretions. Hence, the involvement of IgG<sub>2</sub> in intestinal immunity of calves may begin after the neonatal period.

A more critical evaluation of the immune mechanisms involved in the protection of neonatal calves revealed the importance of specific colostral anti-K antibody of the S-IgA and IgM immunoglobulin classes. The significance of this finding was substantial since neither S-IgA nor IgM anti-K immunoglobulins were present in the whey of the control dams. These results strongly implied that intramammary antigenation stimulated in-situ production of both colostral S-IgA and IgM. It was not infrequently noted that either S-IgA or IgM anti-K would be detected in only one or two quarters of a cow, whereas IgG<sub>1</sub> anti-K was detected more frequently, including in the quarters of the control dams. The extreme variation in the levels of S-IgA and IgM anti-K, among quarters, indicated an autonomous response by udders. Among the vaccine groups concentrations of immunoglobulin classes with anti-capsular activity were not significantly different. However, it was of interest to note that the lowest concentration of S-IgA in the CT group corresponded to the highest scour index in the calves, while the highest concentrations of S-IgA in the KB group corresponded to the lowest scour index for calves.

The values detected for IgM anti-K also related to the scour indices in that the lowest concentration was noted in the whey of CT

dams while the highest concentration was found in the whey of the LB dams. IgG<sub>2</sub> anti-K immunoglobulin was not detected in any of the colostrum whey samples included in these assays.

In addition to the differences in the concentrations of S-IgA and IgM immunoglobulins, among quarters, there was also a marked difference in the occurrence of S-IgA and IgM in quarters of the vaccinated dams. The frequency at which these two immunoglobulins occurred related to the scour index of their respective group. S-IgA was detectable in one-half of the quarters tested in the KB groups while only one-third of the CT group had detectable levels of S-IgA in their whey. The occurrence of IgM was nearly the same for all the vaccine groups which compares to a complete lack of detectable IgM anti-K activity in the colostrum samples of control dams in these studies.

Since anti-O IgG<sub>1</sub>, S-IgA and IgM immunoglobulins are found in high concentrations in the control dams as well as in the vaccine groups, it is not feasible to define the significance of these immunoglobulins in passive protection. The relative values of the immunoglobulin classes are not markedly different among groups, further, they do not relate to the scour indices. Both IgM and IgG<sub>1</sub> anti-O immunoglobulins are present in high concentrations in all of the vaccine groups and noticeably high in the control wheys. S-IgA is not present in near the concentrations of IgG<sub>1</sub> and IgM, although there is

slightly more of this immunoglobulin with anti-O activity and with anti-K activity in whey from all groups. Furthermore, a comparison of the occurrence of anti-O and anti-K antibodies in these three classes, in the whey of all groups, indicates that anti-O immunoglobulins are present in at least 80% of all of the wheys tested, whereas anti-K activity is found much less frequently, i.e., in 0 to 76% of the specimens assayed.

Ingram et al. (1970) showed that low avidity antibodies of the IgM class with activity against the K antigens of E. coli were present less frequently in normal bovine colostrum than were the antibodies with activity against the somatic antigens of the E. coli strains tested. These incomplete antibodies to the K antigens were found in higher titer than were the agglutinating antibodies, whereas little difference was noted between the levels of incomplete antibody and agglutinins against the O antigens. These workers noticed a high degree of cross-reactivity against the O antigens of four different strains of E. coli, whereas only three of the four strains indicated strong cross reactions with the K antigens. It was interesting to note that the three strains possessed the B type K antigen while the fourth less cross reactive strain possessed an A type K antigen.

In field studies and in controlled experiments, the importance of K agglutinins in colostrum and calf serum has been established

(Briggs et al., 1951; Briggs, 1951; Ingram et al., 1953; Ingram et al., 1956; Gay et al., 1964a). The status of the immune activity of the colostrum and serum, however, was based upon the agglutinin content and did not include examination for either the incomplete (natural) IgM antibodies nor for the S-IgA immunoglobulins which are not a highly efficient agglutinating antibody. Low levels of these immunoglobulins could play a significant role in passive immunity to E. coli intestinal infections under field conditions.

It would follow then that the emergence of an E. coli strain characterized by capsular material with more highly refined antigenic determinants could overcome the effectiveness of low avidity (highly cross-reactive) colostral antibody. There is data that indicate that certain K antigens are plasmid controlled and further, that the plasmid can be transferred between strains of E. coli under laboratory conditions (Smith and Linggood, 1972; Ørskov and Ørskov, 1966; Wilson, 1973). It is pointed out that the plasmid controlling K-88 antigens, at least, is transferred independent of the enterotoxin (ent) plasmid in E. coli systems (Smith and Gyles, 1970).

There is adequate evidence in the literature to indicate that the K antigen is involved with the adhesive qualities of certain enteric strains of microorganisms. The role of K antigens in neonatal diarrhea of piglets was recently investigated by Jones and Rutter

(1972). Their results showed that the K-88 antigen of E. coli is responsible for attachment of K-88 positive bacteria to the wall of the small intestine, and that adhesion was essential for the virulence of K-88 positive bacteria in conventionally reared piglets.

In recent studies by Smith and Linggood (1972), an inability to produce diarrhea in lambs with a Kco<sup>-</sup> form of the O8 enteropathogenic lamb strains indicated that common K antigen was concerned with the pathogenic process. The important difference in the bacteriological picture of the lambs given the strain with or without the K antigen was the failure of the negative strain to proliferate in the small intestine. This same strain did proliferate well in ligated intestine, indicating the K antigen of lamb-enteropathogenic strains may function by facilitating adhesion to the intestinal epithelium. Their results further suggested that the adhesive properties may be specific in nature since the pig strain possessing K-88 was unable to adhere properly to calf or lamb intestine while the common K antigen was unable to bring about adhesion to pig intestine.

It was important, therefore, to discover in the present studies that E. coli strain B-44, isolated from an outbreak of bovine colibacillosis could markedly affect the weight gain of neonatal mice. Based upon these findings, an in-vivo assay for testing enterotoxicity of a bovine strain in a laboratory animal was established.

Additionally, the murine model was developed to accommodate investigations of passive immunity against oral challenge with this enteropathogenic strain of E. coli.

Experiments using mice to test virulence of enteropathogenic E. coli of animal origin have been reported by Briggs (1951), Smith and Halls (1968), and Smith and Linggood (1972). These investigators tested the lethality of enteropathogenic E. coli injected intraperitoneally in adult mice. Studies on human enteropathogenic E. coli using neonatal mice were recently reported by Dean et al. (1972). These workers injected broth culture supernatant material into the stomach (intra-gastric) of infant mice and measured the effect of challenge by the ratio of gut weight to body weight.

The system developed for these studies was designed to correlate more closely with the natural conditions in the intestine of a neonatal calf. Therefore, live organisms were administered, per os, to the neonatal mice, and thereafter, the effects were monitored by measuring weight gain over a prescribed period of time. A comparison of weight gain was always made to control animals that had been given sterile broth (negative control) and to neonatal mice given sterile broth and then challenged with the enteropathogen (positive control). The optimum dose required to yield a maximum effect in 4 to 6 day old neonatal mice was determined empirically.

Protection tests were designed to closely resemble the natural conditions in infant calves. This was accomplished by administering colostrum derived antibody by the oral route 4 hours prior to oral challenge with the enteropathogenic organisms.

It was significant that whey from dams, whose calves showed protection in the clinical studies, contributed passive protection to neonatal mice via colostral antibody. These data indicated that neonatal mice could be used to evaluate the efficiency of passive immunity using whey from immunized and non-immunized cows.

It was considered to be of vital importance that 5 of 9 whey specimens from non-immunized dams protected neonatal mice against challenge with B-44. Upon investigation it was noted that even though all of these whey specimens had detectable levels of IgG<sub>1</sub>, IgM and S-IgA anti-O immunoglobulins, the only common immunoglobulin to all of the specimens, with anti-K activity, was IgG<sub>1</sub>. It would appear then that IgG<sub>1</sub>, IgM and S-IgA anti-O in the whey of the control dams was not functional in protecting against the oral challenge of the newborn calf, and furthermore, that the IgG<sub>1</sub> anti-K immunoglobulin only protected the neonatal mice. Further studies of the total influence of the heterocytotropic antibody, IgG<sub>1</sub>, in the murine system are clearly in order. The role of IgG<sub>1</sub> anti-K in the neonatal mouse system could be better ascertained by removing the IgG<sub>1</sub> activity with anti-bovine

IgG<sub>1</sub> serum before passively immunizing the infant mice. An investigation of this nature would be simply done, and it would yield results of a highly significant nature.

In summary, the results of these studies clearly indicate the importance of IgM and S-IgA in passive immunity of neonatal calves in experimentally induced colibacillosis. Of great significance, however, was that IgM and S-IgA with specific activity against the capsular antigens when present in the colostrum of the dam conferred solid immunity to the neonatal calves.

## SUMMARY

Enteric colibacillosis was produced in all of 13 colostrum-fed calves obtained from nonvaccinated dams after calves were given oral challenge inoculation with Escherichia coli strain B-44. Of 9, 6 and 7 calves born to dams given homologous vaccination by the subcutaneous and intramammary route with viable cells (LB), formalin-killed cells (KB) and concentrated crude toxin (CT) preparations, respectively, 7, 6 and 4 calves, respectively, were protected after they were given oral challenge with E. coli.

The disease syndrome included profuse watery diarrhea, severe dehydration, apathy, decrease in body temperature and prostration. The affected calf often died if not treated or if treated too late. E. coli with colony type identical to strain B-44 was detected in the feces of calves from vaccinated and nonvaccinated dams during the 4 days after challenge. Gross pathologic changes were not evident at necropsy.

Colostrum whey contained a heat-labile factor(s) that was involved in the activity measured as O and K agglutinin titers. Heat treatment, 56°C. for 30 min., had a more pronounced effect on antibody activity in colostrum from KB dams than upon titers in the other groups. The heat-lability and specificity of this substance(s) would implicate antibody (natural) of the IgM class.

Agglutinin titers in colostral whey displayed differences, between groups, that were related to the type of vaccine administered to the dam. The immune response to the three vaccines measured as O, K and PHA titers showed the following efficiency; KB>LB>CT>Control. The magnitude of the response (titer) was inversely related to the calf scour index.

Quantitative assays of specific antibody in colostral whey revealed that the IgG<sub>1</sub>, S-IgA and IgM immunoglobulins with anti-O activity were present in the whey of all dams tested, whereas a marked deficiency of S-IgA and IgM anti-K immunoglobulin was noted in whey from control dams only. Scour indices of 0.3, 1.6, 1.9 and 3.7 showed an inverse relationship to the frequency of occurrence and to the levels (mgs%) of S-IgA and IgM in the KB, LB, CT and control groups, respectively.

Immune whey conferred passive immunity in neonatal mice challenged, per os, with the test organism E. coli B-44. Colostrum from some quarters of the control dams was protective in infant mice and appeared to be related to the presence of IgG<sub>1</sub> anti-K immunoglobulin in the colostral whey.

S-IgA and colostral IgM anti-K immunoglobulins were shown to be of signal importance in passive immunity in experimental neonatal bovine colibacillosis.

## APPENDIX

Table A-1. Concentration of Specific Colostral Antibody in Whey from Control Dams.

| Cow Number<br>and Quarter | O-a (1)                    |                 |               | K-a (2)                    |                 |               |
|---------------------------|----------------------------|-----------------|---------------|----------------------------|-----------------|---------------|
|                           | IgG <sub>1</sub><br>(mgs%) | S-IgA<br>(mgs%) | IgM<br>(mgs%) | IgG <sub>1</sub><br>(mgs%) | S-IgA<br>(mgs%) | IgM<br>(mgs%) |
| 413 LF                    | 3                          | 5               | 23            | 0                          | 0               | 0             |
| 413 RF                    | 83                         | 0.7             | 12            | 9                          | 0               | 0             |
| 413 LR                    | 53                         | 0.5             | 10            | 0                          | 0               | 0             |
| 413 RR                    | nd                         | nd              | nd            | 18                         | 0               | 0             |
| 405 RR                    | 8                          | 2               | 72            | 0                          | 0               | 0             |
| 405 LR                    | 58                         | 3               | 47            | nd                         | nd              | nd            |
| 405 RF                    | 52                         | 0.5             | 10            | nd                         | nd              | nd            |
| 427 RF                    | 72                         | 7.3             | 26            | 0                          | 0               | 0             |
| 427 RR                    | 69                         | 0.6             | 13            | 34                         | 0               | 0             |
| 408 RF                    | 26                         | 3.6             | 26            | 66                         | 4               | 0             |
| 408 RR                    | 58                         | 0.6             | 26            | 0                          | 0               | 0             |
| 440 RR                    | 34                         | 2.3             | 3.4           | nd                         | nd              | nd            |
| 440 LF                    | nd                         | nd              | nd            | 18                         | 0               | 0             |
| 440 LR                    | nd                         | nd              | nd            | 58                         | 0.5             | 0             |
| 620 RR                    | 58                         | 4.8             | 46.8          | nd                         | nd              | nd            |
| 620 LR                    | 58                         | 0.5             | 44.8          | 34                         | 0               | 0             |
| 620 RF                    | nd                         | nd              | nd            | 16                         | 0               | 0             |
| 422 LF                    | nd                         | nd              | nd            | 3                          | 0               | 0             |
| 422 RR                    | nd                         | nd              | nd            | 2                          | 0               | 0             |

Abbreviations: LF, Left Front Quarter; RF, Right Front Quarter;  
 LR, Left Rear Quarter; RR, Right Rear Quarter;  
 mgs%, milligrams per 100ml whey;

(1), (2) - See Table 2 in text.

0, none detected

Table A-2. Concentration of Specific Colostral Antibody in Whey from Dams Vaccinated with Crude Toxin.

| Cow Number<br>and Quarter | O-a (1)        |                 |               | K-a (2)        |                 |               |
|---------------------------|----------------|-----------------|---------------|----------------|-----------------|---------------|
|                           | IgG1<br>(mgs%) | S-IgA<br>(mgs%) | IgM<br>(mgs%) | IgG1<br>(mgs%) | S-IgA<br>(mgs%) | IgM<br>(mgs%) |
| 421 LR                    | 16             | 3               | 6             | 0              | 0               | 29            |
| 421 RF                    | 14             | 7               | 14            | 0.2            | 0               | 2             |
| 421 RR                    | 18             | 5               | 4             | 0              | 0               | 0             |
| 421 LF                    | 10             | 3               | 23            | 2              | 2               | 20            |
| 928 LR                    | 20             | 3               | 14            | 2              | 0               | 0             |
| 928 RF                    | 14             | 4               | 20            | 4              | 0               | 0             |
| 928 LF                    | 5              | 3               | 14            | 4              | 3               | 11            |
| 928 RR                    | 4              | 1               | 11            | 4              | 1               | 23            |
| 410 RF                    | 11             | 1               | 2             | 0              | 0               | 0             |
| 410 RR                    | 3              | 2               | 14            | 2              | 5               | 29            |
| 410 LF                    | 5              | 5               | 29            | 0              | 5               | 14            |
| 417 LR                    | 12             | 6               | 23            | 0              | 0               | 0             |
| 417 LF                    | 4              | 3               | 33            | 1              | 0               | 0             |
| 417 RR                    | nd             | nd              | nd            | 0              | 5               | 14            |
| 442 LF                    | 2              | 0               | 0             | 0              | 0               | 0             |
| 442 RF                    | 3              | 0.7             | 6             | 0              | 0               | 0             |
| 442 RR                    | 40             | 3               | 20            | nd             | nd              | nd            |
| 433 RF                    | nd             | nd              | nd            | 52             | 0               | 0             |
| 433 LF                    | nd             | nd              | nd            | 0              | 0               | 0             |
| 433 LR                    | 58             | 1               | 40            | 0              | 0               | 0             |
| 622 LR                    | nd             | nd              | nd            | 16             | 0               | 0             |
| 622 RF                    | nd             | nd              | nd            | 16             | 0               | 0             |
| 622 LF                    | nd             | nd              | nd            | 0              | 0               | 0             |
| 622 RR                    | 17             | 3               | 18            | 0              | 0               | 0             |

Table A-2 continued.

| Cow Number<br>and Quarter | O-a (1)                    |                 |               | K-a (2)                    |                 |               |
|---------------------------|----------------------------|-----------------|---------------|----------------------------|-----------------|---------------|
|                           | IgG <sub>1</sub><br>(mgs%) | S-IgA<br>(mgs%) | IgM<br>(mgs%) | IgG <sub>1</sub><br>(mgs%) | S-IgA<br>(mgs%) | IgM<br>(mgs%) |
| 630 RF                    | 0                          | 3               | 77            | 58                         | 1               | 0             |
| 630 RR                    | 58                         | 3               | 39            | 0                          | 0               | 0             |
| 630 LF                    | 58                         | 2               | 21            | 7                          | 0               | 0             |
| 961 LF                    | 0                          | 1               | 52            | 8                          | 6               | 0             |
| 961 RR                    | 58                         | 3               | 3             | 8                          | 0               | 0             |

Abbreviations: LF, Left Front Quarter; RF, Right Front Quarter;  
 LR, Left Rear Quarter; RR, Right Rear Quarter;  
 mgs%, milligrams per 100 ml whey; n.d., not done;  
 O, none detected

(1), (2) - See Table 2 in text.

Table A-3. Concentration of Specific Colostral Antibody in Whey from Dams Vaccinated with Live Bacteria.

| Cow Number<br>and Quarter | O-a <sup>(1)</sup>         |                 |               | K-a <sup>(2)</sup>         |                 |               |
|---------------------------|----------------------------|-----------------|---------------|----------------------------|-----------------|---------------|
|                           | IgG <sub>1</sub><br>(mgs%) | S-IgA<br>(mgs%) | IgM<br>(mgs%) | IgG <sub>1</sub><br>(mgs%) | S-IgA<br>(mgs%) | IgM<br>(mgs%) |
| 922 RF                    | 15                         | 3               | 0             | 0                          | 0               | 0             |
| 922 LR                    | 12                         | 0.5             | 0             | 0                          | 0               | 0             |
| 922 LF                    | 9                          | 3               | 0             | 0                          | 0               | 0             |
| 922 RR                    | 2                          | 4               | 15            | 31                         | 0               | 0             |
| 650 RF                    | 9                          | 3               | 11            | 2                          | 0               | 0             |
| 650 RR                    | 5                          | 4               | 11            | 0                          | 0               | 0             |
| 650 LR                    | 1                          | 11              | 29            | 10                         | 1               | 0             |
| 429 LR                    | 0.4                        | 7               | 23            | 2                          | 0               | 0             |
| 429 RR                    | 0                          | 4               | 21            | 11                         | 0               | 0             |
| 429 LF                    | 5                          | 2               | 33            | nd                         | nd              | nd            |
| 429 RF                    | 0                          | 4               | 72            | 4                          | 0               | 154           |
| 407 LF                    | 0.2                        | 3               | 20            | 7                          | 1               | 2             |
| 407 LR                    | nd                         | nd              | nd            | 13                         | 2               | 14            |
| 407 RR                    | nd                         | nd              | nd            | 10                         | 1               | 2             |
| 407 RF                    | 0                          | 5               | 38            | 1                          | 0               | 0             |
| 426 LF                    | 4                          | 3               | 33            | 2                          | 3               | 0             |
| 426 RR                    | 4                          | 3               | 0             | 2                          | 2               | 33            |
| 426 LR                    | 93                         | 12              | 46            | 2                          | 0               | 0             |
| 450 LR                    | nd                         | nd              | nd            | 21                         | 6               | 0             |
| 450 RF                    | nd                         | nd              | nd            | 9                          | 9               | 0             |
| 649 RR                    | nd                         | nd              | nd            | 15                         | 6               | 41            |
| 649 RF                    | nd                         | 19              | 46            | 8                          | 6               | 85            |

Table A-3 continued.

| Cow Number<br>and Quarter | O-a <sup>(1)</sup>         |                 |               | K-a <sup>(2)</sup>         |                 |               |
|---------------------------|----------------------------|-----------------|---------------|----------------------------|-----------------|---------------|
|                           | IgG <sub>1</sub><br>(mgs%) | S-IgA<br>(mgs%) | IgM<br>(mgs%) | IgG <sub>1</sub><br>(mgs%) | S-IgA<br>(mgs%) | IgM<br>(mgs%) |
| 937 LR                    | nd                         | nd              | nd            | 8                          | 6               | 77            |
| 937 RR                    | nd                         | nd              | nd            | 0                          | 6               | 0             |
| 403 RR                    | 83                         | 3               | 46            | 3                          | 4               | 0             |
| 403 LR                    | 45                         | 5               | 22            | 0                          | 0               | 0             |
| 403 RF                    | 58                         | 4               | 6             | nd                         | nd              | nd            |

Abbreviations: LF, Left Front Quarter; RF, Right Front Quarter;  
 LR, Left Rear Quarter; RR, Right Rear Quarter  
 mgs%, milligrams per 100 ml whey; n.d., not done;  
 O, none detected

(1),(2) - See Table 2 in text.

Table A-4. Concentration of Specific Colostral Antibody in Whey from Dams Vaccinated with Killed Bacteria.

| Cow Number<br>and Quarter | O-a (1)                    |                 |               | K-a (2)                    |                 |               |
|---------------------------|----------------------------|-----------------|---------------|----------------------------|-----------------|---------------|
|                           | IgG <sub>1</sub><br>(mgs%) | S-IgA<br>(mgs%) | IgM<br>(mgs%) | IgG <sub>1</sub><br>(mgs%) | S-IgA<br>(mgs%) | IgM<br>(mgs%) |
| 618 LR                    | 15                         | 6               | 29            | 5                          | 3               | 14            |
| 618 RR                    | 4                          | 3               | 2             | 5                          | 0               | 0             |
| 618 RF                    | 14                         | 2               | 29            | 4                          | 0               | 0             |
| 618 LF                    | 10                         | 3               | 4             | 4                          | 6               | 29            |
| 921 LR                    | 20                         | 14              | 14            | 10                         | 0               | 0             |
| 921 RF                    | 14                         | 14              | 3             | 9                          | 9               | 0             |
| 921 RR                    | 0                          | 6               | 23            | 14                         | 7               | 0             |
| 921 LF                    | 13                         | 4               | 23            | 15                         | 14              | 51            |
| 444 LR                    | 2                          | 0               | 0             | 10                         | 3               | 14            |
| 444 LF                    | 15                         | 3               | 29            | 7                          | 5               | 6             |
| 444 RF                    | nd                         | nd              | nd            | 10                         | 0               | 0             |
| 439 RR                    | 34                         | 2               | 19            | 0                          | 0               | 0             |
| 439 RF                    | 34                         | 3               | 34            | 0                          | 0               | 0             |
| 439 LF                    | nd                         | nd              | nd            | 0                          | 4               | 46            |
| 948 RR                    | nd                         | nd              | nd            | 0                          | 12              | 9             |
| 948 LF                    | nd                         | nd              | nd            | 0                          | 12              | 0             |
| 404 LF                    | nd                         | nd              | nd            | 10                         | 6               | 0             |
| 404 LR                    | 0                          | 3               | 10            | 4                          | 0               | 0             |
| 437 RR                    | 41                         | 1               | 18            | 0                          | 0               | 0             |
| 437 LR                    | nd                         | nd              | nd            | 16                         | 0               | 0             |
| 437 LF                    | 34                         | 6               | 21            | 16                         | 0               | 0             |

Abbreviations: LF, Left Front Quarter; RF, Right Front Quarter;  
 LR, Left Rear Quarter; RR, Right Rear Quarter, mgs%,  
 milligrams per 100 ml whey; n.d., not done; 0, none  
 (1), (2) - See Table 2 in text. detected.

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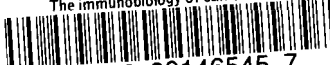
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