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
© Copyright by Richard Adam Wilson (1973)

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 O 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 colostral whey directly reflected the efficacy of the vaccines. The effectiveness of the vaccine preparations was determined to be: KB>LB>CT>Control.

S-IgA and colostral IgM anti-K immunoglobulins were markedly decreased or nondetectable in whey from control dams. The presence of these two immunoglobulins with anti-K activity was strongly related to the degree of protection afforded the neonatal calf.

Neonatal mice passively immunized with bovine colostral 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 colostral IgG1 containing anti-K activity.

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).

THE IMMUNOBIOLOGY OF CALF SCOURS

by

RICHARD ADAM WILSON

A thesis submitted to the Graduate Faculty in partial
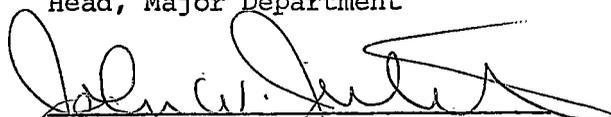fulfillment of the requirements for the degree
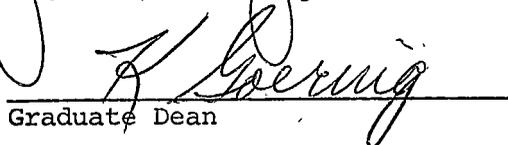
of

DOCTOR OF PHILOSOPHY

in

Microbiology

Approved:

_____
Head, Major Department

_____
Chairman, Examining Committee

_____
Graduate Dean

MONTANA STATE UNIVERSITY
Bozeman, Montana

August, 1973

## ACKNOWLEDGMENTS

iv

TABLE OF CONTENTS

vi

LIST OF TABLES

## LIST OF FIGURES

x

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, $IgG_1$, $IgG_2$, 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 colostral whey directly reflected the efficacy of the vaccines. The effectiveness of the vaccine preparations was determined to be: KB>LB>CT>Control.

S-IgA and colostral IgM anti-K immunoglobulins were markedly decreased or nondetectable in whey from control dams. The presence of these two immunoglobulins with anti-K activity was strongly related to the degree of protection afforded the neonatal calf.

Neonatal mice passively immunized with bovine colostral 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 colostral IgG$_1$ containing anti-K activity.

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 produed 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 endo-toxin portion of the antigen, acts in concert with virulence factors associated with enteropathogenic coliforms.

There is evidence that would implicate K antigens in the viru-
lence of E. coli (Glynn and Howard, 1970; Howard and Glynn, 1971).
These studies indicated that K antigen reduces the complement sensi-
tivity 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 demon-
strated. 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 (Pasieka et al., 1970) and bovine (Wilson et al., 1972) and in the ovine species (Lascelles and McDowell, 1970). A protective role for secretory·immuno-globulins 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 ex-perimentally 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 or-ganism, E. coli B-44, caused severe diarrhea only in calves born to control dams while calves born to immunized dams were strongly pro-tected.

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 anti-body 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, diar-
rhea, 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 creat 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 solublized. 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 concen-

trated 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 concen-

trated 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 result-

ing rabbit anti-SIgA was tested for specificity by reacting the anti-

serum 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 $IgG_1$. 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 $IgG_1$ 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 $IgG_1$ 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 $IgG_1$ 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 $IgG_1$, $IgG_2$,

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% $IgG_1$. The total protein concentration in the solu-

tion was ascertained by absorbance measurements at 278 nanometers.

After making a correction for the amount of $IgG_1$ 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 concen-

trated 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 antigenized 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 $IgG_1$ and $IgG_2$ immunoglobulins to Sepharose beads. Measurements of the protein concentration before and after addition to the column (include all washes) indicated that 80% of the $IgG_2$ and 85% of the $IgG_1$ 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 lypholized $IgG_1$ and $IgG_2$ 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 immunoabsorption. 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 immuno-
diffusion studies. Bovine serum was electrophoresed from a well equi-
distant 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 mono-
specific serum fused with the appropriate band formed by the anti-
bovine serum.

## Quantitation of Immunoglobulins

The procedure used to measure the concentrations of the indi-
vidual immunoglobulins in serum and colostral whey was a modification
of the Masseyeff-Zisswiler technique (Wilson et al., 1972). Ten mm discs
were cut from 1% agarose layered in Hyland Diffusion plates. Anti-
serum 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 concen-
tration 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 concen-
tration 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 concentra-
tion of the working standards of purified immunoglobulin solutions
were calculated from optical densities measured at 278 nm.  The ex-
tinction coefficients used in these experiments were 13.8 for $IgG_1$,
$IgG_2$, IgA and 12.8 for IgM.  The curves indicate that a linear rela-
tionship exists between the concentrations of antigen and the diameter
of the precipitin rings.  Furthermore, the concentration of the anti-
gen 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 activ-
ities of $IgG_1$, $IgG_2$, 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. colostral 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 amino-acetic 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 immuno-diffusion 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 enterotoxic 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. Enterotoxic 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 eosphogeal 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:

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

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

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

## 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 con-

centrated 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 I). 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 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) | | |
| 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 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 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 inter-
vals.  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.

| Experimental group [f] | Anti-O [a] | | Anti-K [b] | | PHA [c] | |
|---|---|---|---|---|---|---|
| | Two weeks post-injection | At parturition [e] | Two weeks post-injection | At parturition [e] | Two weeks post-injection | At Parturition [e] |
| C | $<10^d$ | $<10^d$ | $<10^d$ | $<10^d$ | $930^d$ | $1274^d$ |
| 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 | LB | 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 [f] | Anti-O [a] | | Anti-K [b] | | PHA [c] | |
|---|---|---|---|---|---|---|
| | At birth | At four days of age | At birth | At four days of age | At birth | At four days of age |
| C | $<10^d$ | $<10^d$ | $<10^d$ | $<10^d$ | $<40^d$ | $24^d$ |
| 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.

Colostral 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).