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INDIGESTION IN YOUNG CALVES VI

STUDIES OF DIARRHEIC CALVES FED MILK REPLACERS MADE OF NORMAL AND HEAT-TREATED SKIM MILK AND WHEY POWDER*

By

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LANDSVERK, T., B. LAKSESVELA, E. LIVEN, A. LUND, Ø. ØDEGAARD and I. ØRSTAVIK: *Indigestion in young calves. VI. Studies of diarrheic calves fed milk replacers made of normal and heat-treated skim milk and whey powder.* Acta vet. scand. 1983, *24*, 431—445. — Diarrhea occurred in 12 pre-ruminant calves after the introduction of milk replacers. Six of the calves were fed a replacer made of normally treated skim milk and whey powder, while the other 6 got a replacer made of heat-treated powders, although otherwise similar to that of the first group. Abomasal curd formation was deficient in calves fed for 9 or 17 days on the heat-treated replacer (4 of 6 calves). Otherwise, no clear differences between the calves given normally treated and heat-treated replacer occurred, as regards diarrhea, bacteriological, virological, and pathomorphological findings in the intestinal tract. Rotavirus and chlamydial infections were indicated and considered as the major cause of the diarrheic condition, although bacteria, including coliforms and Pseudomonas aeruginosa, may have contributed. The milk replacers may have been involved was provoking factors.

heat-treatment; skim milk; whey; diarrhea; curd formation; intestinal microflora; rotavirus; chlamydia; calves.

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In our first study with heat-treated skim milk and whey powder (*Laksesvela et al.* 1978) conflicting results were obtained regarding the influence of heat-treatment of powders on calf diarrhea. Heat-treatment was associated with an increase in the frequency of diarrhea in the first experiment but little in the second, and it was questioned whether the calves in the 2 experiments might have met with a different microflora or if other factors were involved. In other reports heat-treatment of powders has been associated with increased frequency of digestive upsets (*Shilliam et al.* 1960, *Lister & Emmons* 1976), however, microbial factors possibly contributing to the condition have been poorly studied.

The present paper describes an experiment similar to the previous one (*Laksesvela et al.*); in this study, however, more extensive microbiological and morphological examinations have been carried out. A description of enteropathogenic agents not previously reported in Norway is also included. This paper is complementary to one focusing on the pathomorphological aspects of the experiment (*Landsverk* 1981b).

MATERIALS AND METHODS

Animals and diets

The calves and diets are set out in Tables 1 and 2. Calves 1—6 on diet B1 and 7—12 on diet B2 were obtained from a number of herds in the neighbourhood of Oslo. The calves were of Norwegian Red Cattle breed (NRF) and had been reared with colostrum at birth and thereafter with whole cow's milk (WM) twice daily at a rate of 110 ml/kg body weight/day. After 1 week at the Research Station, Holt, the calves were introduced to the experimental diets. The calves then ranged from 7—21 days of age. On the first day on experimental diet the milk replacer was mixed with an equal amount of WM. The whey and skim milk powders were reconstituted with water at a temperature of about 35°C to contain 10.3 % dry matter, and the replacer was bucket-fed twice daily at 8 am and 4 pm at a rate of 110 ml/kg body weight/day. The calves were euthanized at different intervals from the start of the feeding on milk replacers (Table 2).

Calves 13, 14 on diet B3 and 17—21 on WM were born and kept throughout their lives on the Research Station, Heggedal. The calves on B3 were reared in the same manner as those on B1

Table 1. Composition and nutrient content of diets.

Diet	Calves No	Quantity in liters/day/ 10 kg calf	Dry matter concen- tration	Skim milk powder %	Whey powder %	Lactose % of dry matter	Protein % of dry matter	Fat % of dry matter
WM	17—21	1.1	12.2	—	—	36.6	26.5	31.2
B1, B2	1—12	1.1	10.3	39.7	40.0	50.8	19.7	20.9
B3	13, 14	1.1	10.3	38.8	38.8	50.7	20.2	21.0

WM whole cow's milk.
B1 and B2 milk replacer with emulgated butter as fat source, without and with heat treatment of powder.
B3 milk replacer with cream as fat source, normally dried powder.

On diets B1—B2 the milk replacers were supplemented with 0.8 % refined lecithin and synthetic monoglyceride in a ratio of 82:18. Calves on diets B1, B2 and B3 were given a pre-mix supplying the following per kg air dry powder: Vit. A 20,000, Vit. D 30,000, Vit. E 30, all in i.u.

Table 2. Experimental details on the calves.

Calf No	Sex	Diet	Days on exp. diet	Age at euthanasia (days)
1	♂		3	21
2	♂		3	10
3	♂	B1	9	27
4	♂		9	24
5	♂		17	33
6	♂		17	29
7	♂		3	21
8	♂		3	10
9	♂	B2	9	21
10	♂		9	25
11	♂		17	30
12	♂		17	31
13	♀	B3	5	26
14	♂		5	18
17	♀		—	20
18	♀		—	21
19	♂	WM	—	17
20	♂		—	22
21	♀		—	23

and B2. Experimental details for calves 17—21 have been with previously (*Landsverk* 1979, 1980).

The whey and skim milk powder used in diets B1 and B3 were vacuum condensed and spray dried at low temperature. The heat-treated products used in diet B2 were subjected to 80°C for 45 min in a condensed state and were subsequently treated as previously described (*Laksesvela et al*). The effect of the heat-treatment was estimated by the content of available lysine analyzed by the method of *Rexen & Christensen* (1960)*. Available lysine expressed as g/16g N was: Normally treated skim milk powder 6.79, heat-treated skim milk powder 5.40, normally treated whey powder 5.20 and heat-treated whey powder 3.26.

The calves were kept on metal or wooden grates in individual pens. No bedding material was used.

Bacteriological examination

Prior to the introduction of the milk replacers to the experimental animals fecal samples were examined for the presence of Salmonella spp. employing standard bacteriological procedures including enrichment for 3 days on potassium tetrathionate broth.

After euthanasia interstinal content from the rumen, anterior, middle, posterior small intestine and colon was collected for bacteriological examination. Qualitative and quantitative examinations were carried out on blood agar, enterococcus agar (Difco) and M.R.S. (lactobacillus) agar. Characterization of organisms cultivated on blood agar (coliforms) was based on morphological, cultural and biochemical properties. Samples from calves 18—21 were examined qualitatively only. The blood agar plates were incubated aerobically for 24 h at 37°C. The enterococcus and M.R.S. agar plates were incubated in 5 % CO_2 for 72 h at 37°C.

Fluorescent antibody techniques

Detection of virus antigen. Rota- and coronavirus immune serum fluorescein-labelled conjugates were received from the National Institute for Veterinary Research, Brussels, Belgium. The conjugates appeared in a freeze dried state and were reconstituted to working dilutions (1:32 for rota and 1:16 for coronavirus) with phosphate buffered saline (PBS). Evans

* The analysis was carried out at Bioteknisk Institut, Kolding, Denmark.

blue at a final concentration of 1:100,000 was added to the conjugates before use. The reactions were left to occur on frozen intestinal sections cut at about 8 µ. Samples from the middle duodenum (d2), middle jejunum, posterior jejunum 3 (pj3) and colon were examined. The sections were post-fixed at 4°C in acetone for 30 min, air dried, flooded with conjugate, incubated in a dark and moist chamber for 1 h at room temperature, washed 3 times, each for 5 min in PBS, and mounted in equal amounts of glycerol and PBS. The sections were examined immediately in a Zeiss standard incident light fluorescent microscope.

Control procedures included an indirect immunofluorescent test using unconjugated rabbit positive and negative antiserum to calf rotavirus obtained from the Microbiological Laboratory, Ullevål Hospital, Oslo. These sera were diluted 1:40. Sections positive and negative for rotavirus antigen were fixed as above and incubated with negative or positive antisera for 30 min. The sections were washed 3 times in PBS and then incubated with FITC conjugated goat anti-rabbit globuline*, diluted 1:20, with the addition of Evans blue as above, for 30 min. The further treatment was as above.

S e r o l o g y. The sera were collected before euthanasia and tested for antibodies against a bovine strain of rotavirus (BDV-27) by indirect immunofluorescence, essentially as described by *Örstavik et al.* (1976). The antigen consisted of virus infected BSC-1 cells. Fluorescein labelled rabbit anti-bovine globuline (Wellcome Reagents Limited, London) was used at the working dilution 1:30.

Morphological examination

Intestinal specimens were sampled for light microscopical (LM), scanning electron microscopical (SEM), and transmission electron microscopical (TEM) examination. The methods have been described (*Landsverk* 1979, 1981a).

RESULTS

Clinical examination

Although the results from the clinical examinations have been given elsewhere when dealing with other aspects of the experiment (*Landsverk* 1981b), some selected data, necessary for the

* Goat anti-rabbit globuline GIBCO, K78, A976604.

context of the present paper are given: Calves 13, 14 on B3 and 17—21 on WM appeared healthy throughout their lives. Calves 1—6 on B1 and 7—12 on B2 got diarrhea after the introduction of the milk replacers. No difference in the frequency of diarrhea between calves on diets B1 and B2 was found (Table 3). After 11—12 days with diarrhea the 4 remaining calves not already euthanized (calves 5, 6, 11, 12) showed clinical improvement. Although included among the affected calves, these calves (5, 6, 11, 12) also are referred to as convalescents in the later description.

Table 3. Selected clinical and microbiological results.

Calf No	Diet	Days with diarrhea before euthanasia	Clinical status at euthanasia	Immuno-fluor. rota-virus intestine	Antirota-viral titers in serum	Chlamydial agents intestine (LM, TEM)
1		1/3	D	+	1:10	0
2		1/3	D	0	1:10	0
3	B1	5	D	0	1:10	+
4		3	D	0	<1:10	0
5		12	C	0	1:320	+
6		11	C	0	1:320	0
7		3	D	0	<1:10	0
8		1	D	+	ND	0
9		5	D	0	1:20	0
10	B2	7	D	0	1:40	0
11		12	C	0	1:40	+
12		12	C	0	1:160	+
13,14,17—21	B3, WM	—	N	0	1:10—<1:10	0

N = normal, no diarrhea; D= diarrhea, C = convalescent; ND = not done; LM = light microscopy; TEM = transmission electron microscopy.

Bacteriological examination

Increasing numbers of coliforms, lactobacilli and enterococci were found in the intestinal contents from the anterior to the posterior portions of the intestine of all calves. No clear differences between calves on B1 and B2 were found. On the other hand, there seemed to be a tendency to higher bacterial counts in the affected calves (1—12) than in the healthy ones (calves

Figure 1. Immunofluorescence, FITC rotavirus conjugate, middle jejunum, diarrheic calf (1). Positive fluorescence in epithelial cells of the villous tip. × 150.

13, 14, 17). The numbers of coliforms and Pseudomonas aeruginosa being of particular interest, are specified below:

The number of coliforms in the anterior small intestine was relatively low except for calf 7 on B1 and calf 14 on B3, having counts 7.8 and 5.8 ($\log_{10}$ organisms/ml intestinal content), respectively. For the rest of the calves the counts were < 5.0. In the middle jejunum calves 1,4,5 on B1 and 7,10 on B2 showed counts of coliforms within the range of 6.0—6.8; other calves had counts < 5.0—5.9. For calf 1 microscopical examination revealed large numbers of Gram negative rod-shaped bacteria covering the tips of the villi. In the posterior small intestine of calves 1,4,5,6 on B1, 10,12 on B2, and 13 on B3 the number of coliforms amounted to 6.6—7.8 whereas in the other calves the counts were < 5.0—6.5. The counts in colon were uniformely high, in most calves 7.0—8.0.

Pseudomonas aeruginosa was present in calves 5, 6 on B1 and 9, 11 on B2. The count varied within the range of 4.6—7.1, the highest numbers being found in the posterior small intestine and colon. Although the complete results for the examinations of the ruminal contents will not be described in the present paper, it

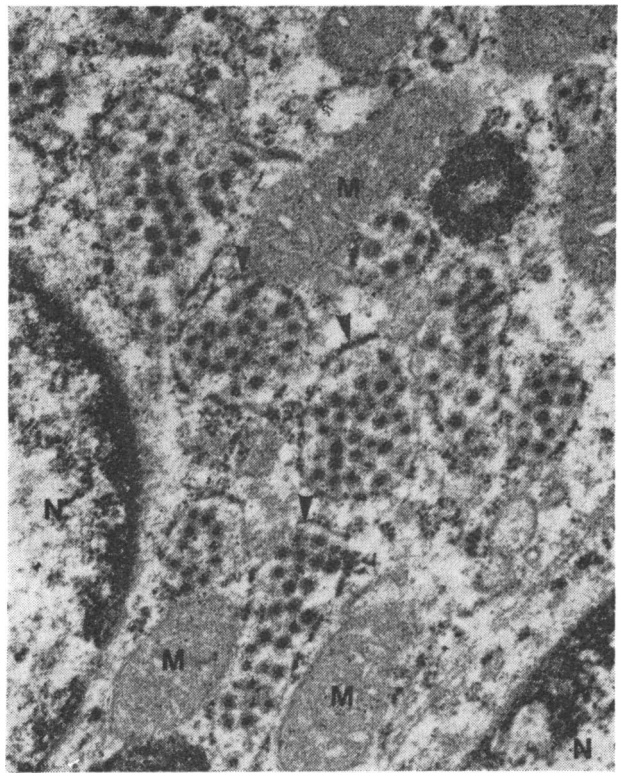


Figure 2. Transmission electron micrograph, diarrheic calf (1). Virions within dilated endoplasmic reticulum (arrows) in an epithelial cell at the villous tip. The virions consist of a dense nucleoid surrounded by a less dense outer coat, M = mitochondria, N = host cell nuclei. × 51,000.

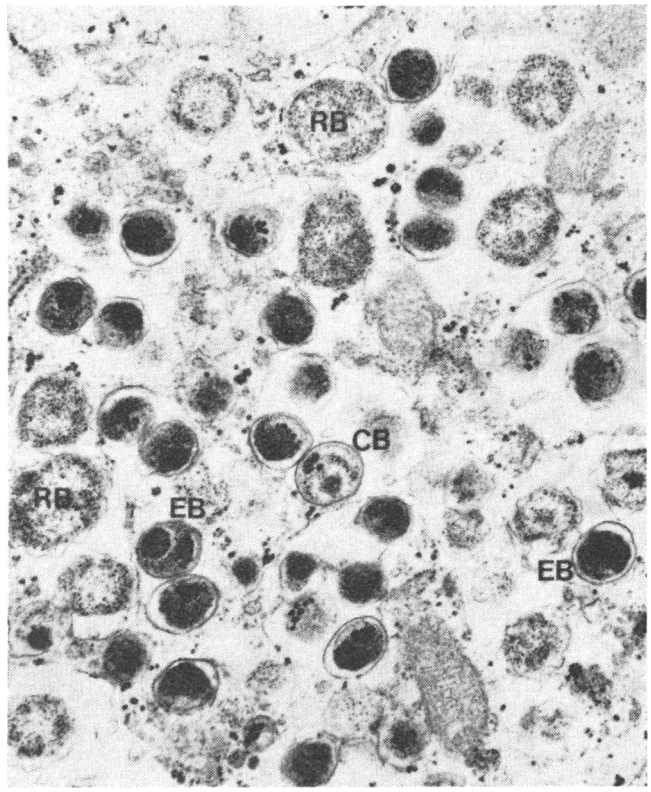
may be appropriate to mention that calf 9, which showed severe rumenitis and enteritis, had Pseudomonas aeruginosa at a number of 9.0, whereas the other calves had counts < 7.0 in ruminal content.

Examinations for Salmonella spp. were negative in all samples.

Fluorescence microscopy

Detection of rotavirus antigen. Results form the immunofluorescence test on frozen sections are given in Table 3.

Calves positive for rotavirus (1 and 8) showed fluorescence in apical villous epithelial cells of duodenum, middle- and posterior jejunum (Fig. 1). The direct and indirect immuno-



F i g u r e 3. Transmission electron micrograph, diarrheic calf (3). Chlamydia within cytoplasmic vacuoles of a follicle-associated epithelial cell over Peyer's patches. The chlamydia are in different stages of development; EB = elementary bodies, CB = condensing body, and RB = reticulate bodies. × 33,000.

fluorescence procedures gave the same results. Control positive sections stained with rabbit serum negative for rotavirus antibodies were negative in the indirect procedure.

S e r o l o g y. The results of the examinations for serum antibodies against rotavirus are given in Table 3.

Gross and microscopical changes

These results have to a great extent been dealt with elsewhere (*Landsverk* 1981b). Selected aspects of interest in the present context may be referred to: The calves were in the pre-ruminant stage as judged by the lack of development of the forestomachs. Abnormalities in the forestomachs were mostly absent except for

the frequent occurrence of hair bezoars and rumenitis in calf 9. Inspection of abomasal curds performed at necropsy revealed that these were usually of 1—10 cm in diameter. Calves 9—12 on the heat-treated replacer B2, however, showed lack of curds and had a porridge-like abomasal content.

Gross changes of the intestines in the affected calves (1—12) were mostly restricted to moderate congestion except for the change of colon content corresponding to the diarrhea. Calf 9 represented an exception showing severe congestion and edema of the intestines, mesenterium and mesenterial lymph nodes. LM including morphometry demonstrated intestinal villous atrophy and crypt elongation in all calves 1—12; no significant difference between calves on diets B1 and B2 was found. SEM confirmed villous stunting. In calf 1 large numbers of rod-shaped bacteria covered the tips of the villi in the middle jejunum. TEM confirmed the results from the immunofluorescence studies, demonstrating viral particles about 65 nm in diameter within dilated cisterna of the endoplasmic reticulum in apical villous epithelial cells (Fig. 2), the morphology corresponding to that of rotavirus (*Stair et al.* 1973). LM and Macchiavello's stain revealed chlamydia-like inclusions in the follicle-associated epithelium over Peyer's patches in some of the calves (Table 3). The identity of these structures was confirmed by TEM (Fig. 3), revealing features suggested as specific for chlamydia (*Doughri et al.* 1973, *Todd et al.* 1976).

DISCUSSION

The present experiment did not confirm any significant difference between heat-treated and normally treated powder in milk replacers for calves as regards to diarrhea and microbiological/pathomorphological changes. In contrast, other authors report increased incidence of diarrhea with heat-treated powder. In the present experiment diarrhea occurred in all calves in both groups after the introduction of the milk replacers and one might speculate as to the possible contribution from constituents common to the replacers B1 and B2. Actually, the composition of the replacers, and especially the inclusion of whey powder, resulted in a moderate increase of the lactose content as compared with whole cow's milk (from about 245 to about 285 g/day/50 kg calf) and increased levels of lactose have been associated with diarrhea (*Rojas et al.* 1948, *Blaxter & Wood* 1953, *Walker & Faichney*

1964, *Slagsvold et al.* 1977). However, it is apparent from the lack of diarrhea on the almost similar diet B3 that the lactose levels reached on diets B1 and B2 could not alone be responsible for the condition.

In vitro studies have indicated poor curd formation with replacers made of heat-treated milk powder (*Tagari & Roy* 1969, *Emmons & Lister* 1976). In the present material the calves fed for 9 or 17 days on the heat-treated replacer showed absence of curds apparently confirming the above in vitro results. In fact, this seems to be the first in vivo demonstration of poor curd formation with a heat-treated replacer. The formation of the abomasal curds is of importance in the digestive function of the calf, providing a sustained release of the abomasal contents (*Mylrea* 1966) probably beneficial to intestinal digestion and absorption of nutrients in the calf. The formation of an abomasal casein clot also seems advantageous to abomasal protein breakdown. In early life the calf seems dependent on the abomasal enzyme rennin in the initial stages of protein breakdown and this enzyme is to a large extent specific for the breakdown of casein (*Preston* 1964). Actually, the deficient curd formation with heat-treatment of powder may be one of the causes for the diminished digestibility of the milk proteins reported on such diets.

Undigested proteins may influence the intestinal microflora in providing a substrate for bacteria and causing proliferation of these. Such a mechanism has been proposed as an explanation for the frequent digestive upsets in calves on heat-treated diets (*Roy* 1969). However, the results from the present experiment do not give support to the above concept, rather, factors other than heat-treatment seem to have been most important.

On the other hand, the bacteriological findings support an earlier report on an anterior - posterior gradient for bacteria in the intestine, not much different in diarrheic and healthy calves (*Smith* 1962). Only 1 calf showed bacteria adhering to the villous surface, coinciding with high numbers of coliforms at this site. These organisms could be enteropathogenic Escherichia coli. Pseudomonas aeruginosa may likewise have been of pathogenic significance, being found in some of the diarrheic but not in the healthy calves.

Rotavirus and chlamydial infections, however, were probably of more decisive importance to the development of the present

diarrheic condition. Although rotavirus agents were demonstrated in 2 calves only, the serological data indicated the probability of a widespread rotavirus infection among the affected calves 1—12. The chlamydial infection may also have been more widespread than indicated by the direct demonstration of agents. Chlamydia showed a marked predilection for the follocle-associated epithelium (FAE) over Peyer's patches and were associated with lesions in this compartment including fusion of the FAE with the absorbing epithelium (*Landsverk* 1981c). The high prevalence of this lesion might be related to a chlamydial infection, assuming that in some cases the agents may have disappeared from the epithelial cells. However, the specificity of the lesions and the extent to which other agents may contribute to the lesions remain to be elucidated.

The outbreak of diarrhea occurred after the introduction of milk replacers, a fact which seems to indicate that the diet or possibly the change in diet, may have been one of the factors provoking the diarrhea. The complexity of the diarrheal problem in calves probably involves a number of cooperating factors such as the immune status of the calves, transport, diet, environment, and the occurrence of enteropathogenic agents (*Roy* 1964, 1969, *Bywater* 1975, *Appleman & Owen* 1975, *Simensen et al.* 1980). It may be the sum of these various factors that determines the actual outcome for the individual calf. In the present context the influence of the heat-treatment of powder might just not have been decisive enough or, rather, other factors may have been dominant so that differences between diets B1 and B2 were not manifested.

The present study clearly demonstrates the advantage of including microbiological examinations in experimental studies of this type. Apart from elucidating the factors operative during diarrhea, the results in the present study also give information about agents of calf diarrhea in Norway. The present observations of rotavirus and chlamydia in association with calf diarrhea are the first ones in this country, although rotavirus has previously been demonstrated connected with diarrhea of man (*Ørstavik et al.* 1976), and antibodies have been demonstrated by immunfluorescence in sera from randomly selected cattle (*Ødegaard*, unpublished). However, in other countries both agents have been incriminated as factors of importance in calf diarrhea (*Mebus et al.* 1971, *Doughri et al.* 1974, *Morin et al.* 1974, *McNulty et al.*

1976, *van Opdenbosch et al.* 1979). The present findings emphasize the need for a further investigation of the role of infections in calf diarrhea in Norway.

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SAMMENDRAG

Indigestion hos unge kalver. VI. Undersøkelser av diarékalver fôret med melke-erstatninger laget av normal- og varmebehandlet tørrmelks- og mysepulver.

Diaré opptrådte hos 12 unge kalver etter at de var satt på en melke-erstatningsdiett. Seks av kalvene fikk en erstatning laget av normalbehandlet myse- og tørrmelkspulver, de andre 6 fikk en tilsvarende diett, men hvor pulveret var varmebehandlet. Fire kalver som hadde fått varmebehandlet erstatning i henholdsvis 9 og 17 dager manglet løpekoagler. Ellers var det ikke sikre forskjeller mellom gruppene, hverken med hensyn til kvantitativt bakteriologiske, virologiske eller patomorfologiske funn i tarmkanalen. Rotavirus og chlamydier ble påvist hos noen av kalvene og betraktet som vesentlige årsaker til diaréen, selv om også koliforme bakterier og Pseudomonas aeruginosa kan ha bidratt. Melke-erstatningene kan ha spilt en rolle som provoserende faktorer.

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