USE OF HYDROGELS TO VACCINATE CALVES ORALLY FOR PNEUMONIC PASTEURELLOSIS

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Introduction

Pasteurella haemolytica is the most common bacterium that causes death in cattle affected by Bovine Respiratory Disease Complex. The newest vaccine to prevent pneumonic pasteurellosis is composed of culture supernatants(1). Culture supernatants are inexpensive to produce, and do not cause disease. However, the efficacy of this vaccine has been questioned(2). As with other vaccines used to prevent pasteurellosis, this vaccine has been used parenterally in feedlots and sales barns where cattle have already been stressed and exposed to multiple pathogens. Vaccination prior to exposure is desirable ie., when calves are still on the farm of birth on pasture. The most practical way to vaccinate these calves is through the feed or water.

Oral vaccination would stimulate the gut-associated lymphoid tissue (GALT). Migration of lymphocytes from GALT results in increased immunity at other mucosal sites including the lung (3). Immunity to a variety of infectious agents, including Streptococcus mutans, influenza and Sendai viruses, and cholera toxin has been induced at other mucosal surfaces in laboratory animals and man following oral administration(4-7). The possibility of orally administered vaccines in cattle has not been investigated until recently. Studies have shown that culture supernatants (CS) of P. haemolytica introduced into the intestine stimulate an immune response in the lungs of cattle(8). However, the oral administration of vaccines is much more difficult to achieve in ruminants.

Oral vaccination of cattle requires that the antigen be able to withstand the extreme changes in pH and severe action of proteolytic enzymes in the upper gastrointestinal tract (GIT). Cattle have a complex upper GIT composed of 4 stomachs. Bypassing the rumen or first stomach is especially demanding since bacterial degradation would also be likely to destroy most antigens. A carrier is needed that can protect antigens until they reach the lower GIT. Polymethacrylic acid hydrogels have been used to deliver drugs over time to animals (9). We have demonstrated that hydrogels can bypass the rumen to deliver a model antigen to the lower GIT and release antigen for 96 hours(10). In this study we hypothesized that hydrogels could deliver culture supernatants of P. haemolytica in an oral vaccine to the GALT of cattle resulting in protection against pneumonic pasteurellosis.

Materials and Methods

Loading hydrogels with culture supernatants - In a previous study hydrogels were loaded with chromium

EDTA which was released in a sustained release manner(10). Culture supernatants (CS) contain many antigens, including a proteinaceous exotoxin (leukotoxin) 102 kd in size. Therefore, it was necessary to determine whether CS could be loaded and released from hydrogels. Hydrogels were produced as previously described(10). Gels were loaded with CS which had been harvested from P. haemolytica cultured to the active phase of growth, lyophilized, and resuspended to a 22% (w/v) solution. Loaded gels were dried to a hard glassy consistency by placing them in a 37°C incubator for 48 hours. The dried hydrogels were then ready to administer as an oral vaccine.

Antigen release studies in vitro - To test for release of the CS antigens, 3 loaded hydrogels were placed in saline and allowed to hydrate. Eluent was removed from the hydrogels daily for 3 days and replace by fresh saline. The eluents were tested for the presence of leukotoxin, the primary protein antigen present in CS, by sodium dodecyl sulfate-polyacryamide gel electrophoresis (SDS-PAGE) analysis and enzyme linked immunosorbent assay (ELISA). Eluents were concentrated by precipitation, denatured by boiling in buffer with sodium dodecyl sulfate for 5 minutes, and subjected to electrophoresis. A sample of CS was used as a positive control. The gel was stained with Coomasie blue and the molecular weight of the bands determined. ELISA was performed by binding 50 ul of each eluent to the well of an Immulon 2 (Dynatech Laboratories) polystyrene plate overnight at 4°C. A polyvalent rabbit antibody made in our laboratory to the 102 kd leukotoxin of P. haemolytica was used to detect the presence of leukotoxin in eluents. Secondary antibody was horseradish peroxidase conjugated rabbit anti-bovine IgG (Bethyl Laboratories Inc.) Orthophenyldiamine (Sigma) was used as substrate. The reaction was stopped after 30 minutes using sulfuric acid and the plate was read using an EIA spectrophotometer (Molecular Devices Corp.).

Calf vaccine trial - Twelve Holstein-Friesian 4 month old calves were divided into 2 groups. Experimental calves (vaccinates) were given 300 CS-loaded hydrogels per day placed inside two 15 ml gelatin boluses for 5 days by balling gun. Control calves (non-vaccinates) were given 300 plain hydrogels. Pulmonary lavage was performed prior to vaccination and 2 weeks after the first oral dose of hydrogels. Serum was collected on the same days as the lavages. Three weeks after the first day of vaccination each calf was challenged with an intrabronchial inoculation of 25 ml of 10^9/ml of virulent P. haemolytica. Calves were monitored for clinical signs of disease. Calves which survived for seventy-two hours were euthanatized and a post mortem examination performed. Lungs were scored for the percentage of pneumonic lesions, gross and histopathological lesion score, and a pneumonic index was computed by multiplying the first 3 values together. Scores for calves were ranked by survival time and pneumonic index and the data analyzed using the Wilcoxon rank sum statistical test.

Specific isotypic antibodies to the CS of P. haemolytica were assayed in serum and pulmonary lavage fluids by ELISA. The CS were absorbed to the plate as described above and the samples added, serum at a 1:100 dilution and lavage fluids at a 1:10 dilution. Monoclonal antibody to a specific bovine isotypic antibody (VMRD, Inc.) was added followed by biotin labelled rabbit anti-mouse antibody. Horseradish peroxidase labelled avidin was added followed by OPD substrate as described above and the plate read on an EIA reader. The mean absorbance for each antibody isotype was measured

in serum in vaccinated and non-vaccinated calves and post vaccination values compared using the student t-test. For pulmonary lavage samples, the total antibodies present were also assayed using anti-bovine antibody in order to determine the ratio of specific antibody to total antibody for pre- and post-vaccination samples. The ratio of post to pre-ratios was determined and compared by student t-test for each antibody isotype in vaccinated and non-vaccinated calves.

Results and Discussion

Antigen release studies - One major protein band was noted in the SDS-PAGE analysis of eluents at 102 kd, the molecular weight of the leukotoxin (data not shown). In the ELISA assay the absorbance values for the eluents indicated the presence of leukotoxin in eluents collected over 3 days (Table 1). These results suggested that the hydrogels loaded with CS were appropriate to test in calves.

Calf challenge studies - The survival time post-challenge in hours, per cent pneumonic lung, gross and histopathological lesion scores for each calf is shown in Table 2. There was a significantly lower percentage of pneumonic lung, lower gross lesion score, lower histopathological lesion score, and pneumonic index combined with survival time for vaccinated calves compared to non-vaccinates ($p<.05$) as shown in Table 3. Wilcoxon rank sum analysis demonstrated that vaccinated animals had significantly less lesions and greater survivability than non-vaccinated controls ($p=.045$).

Immunoglobulin titers - There was an increase in CS specific IgM, IgG_1, and IgA ($p = .075$) in pulmonary lavage fluids in vaccinated calves compared to non-vaccinates (Table 4). There was no change in any CS specific serum antibody isotypes in vaccinated calves. These results suggest a primary mucosal stimulation occurred. A greater immune response may have been detected had the calves been administered subsequent inoculations either orally,

TABLE 1

ELISA results of leukotoxin eluted from gels hydrated in phosphate buffered saline for 3 days.

Sample Tested	Absorbance Reading
Culture supernatant used to load gels	.421
Tryptic soy broth	.207
Eluent day 1	.391
Eluent day 2	.351
Eluent day 3	.468

Table 2

SURVIVAL AND POST-MORTEM RESULTS OF CALVES FOLLOWING CHALLENGE BY P. HAEMOLYTICA

Trial	ID	Treatment Group	Survival (hours)	Percentage Pneumonic Lung	Gross Lesion Score	Histopath. Lesion Score	Pneumonic Index
1	74	V	3.5	0.4	6.0	3.5	8
1	77	V	3.5	2.1	6.0	5.8	71
1	78	V	23*	25.8	12.5	15.8	5105
1	75	C	3.5	53.7	15.0	6.2	4994
1	76	C	3.5	61.4	13.5	7.7	6349
1	80	C	72**	24.2	17.0	4.4	1810
2	82	V	72**	44.2	9.5	1.2	487
2	84	V	72**	28.8	8.5	0.9	211
2	87	V	72**	31.3	10.5	1.6	526
2	81	C	12	100	11.0	3.5	3850
2	83	C	12	100	10.0	6.8	6830
2	85	C	20	100	10.0	2.5	2500

C = non-vaccinated control animals
V = vaccinated animals
* = euthanized because moribund
** = euthanized at end of trial
Gross lesion score was based on the severity of parameters (fibrin, edema, consolidation, etc) with a maximum score of 24 for the most severe lesions. Histopathological lesion score was based on the severity of 14 different parameters (necrosis, fibrin, etc) with a maximum score of 42 for the most severe lesions. Pneumonic index was computed by multiplying the percentage pneumonic lung x gross lesion score x histopathological lesion score.

Table 3

RANKING WITHIN TRIAL BY SURVIVAL AND PNEUMONIC INDEX

Trial	Rank	ID	Survival	Pneumonic Index	Treatment Group
1	1	76	3.5	6349	C
1	2	75	3.5	4994	C
1	3	77	3.5	71	V
1	4	74	3.5	8	V
1	5	78	23	5105	V
1	6	80	72	1810	C
2	1	83	12	6830	C
2	2	81	12	3850	C
2	3	85	20	2500	C
2	4	87	72	526	V
2	5	82	72	487	V
2	6	84	72	211	V

1. C = non-vaccinated control animals
   V = vaccinated animals
2. Key: rank 1 = worst rank 6 = best
3. Survival + Percent Pneumonic Lung $P = .040$
   Survival + Gross Lesion Score $P = .035$
   Survival + Histo. Lesion Score $P = .045$
4. In trial 2, all C's rank above all V's. In trial 1, only one C breaks this pattern. For both trials together, the exact P-value for the rankings is $P = .045$; thus there is statistically significant evidence ($P < .05$) that vaccinated animals had less severe lesions following challenge.

Table 4
Antibody Response in Calves

	Pulmonary		Serum	
	Vaccinate	Control	Vaccinate	Control
IgM	1.60*	1.04	1.491**	1.510
IgG1	1.83	1.46	.417	.408
IgG2	1.01	1.08	.041	.050
IgA	1.25+	0.78	.011	.017

BAL = bronchoalveolar lavage
* Ratio of specific antibody/total immunoglobulin post-vaccination to the same ratio pre-vaccination. Vaccinates were vaccinated for 5 days by oral inoculation of culture supernatants absorbed into polymeric beads. Control calves were given plain beads. Post-vaccination BAL was performed 2 weeks after first oral dose was given which was the same day pre-vaccination BAL was performed.
\+ p = 0.075
** Mean absorbance value of serum 2 weeks post-vaccination as determined by ELISA using culture supernatants as antigen.

intranasally, or parenterally. Enhanced immune responses at mucosal sites have been documented for orally primed animals (11).

Passage of hydrogels - Feces were examined during the time calves were being vaccinated for the presence of hydrogels. Five hydrogels, mildly hydrated to non-hydrated, were found in feces. At post mortem the entire GIT was examined and no hydrogels were found. This suggests that most hydrogels were probably retained in the reticulum and were eroded due to the extremely coarse consistency of the ingesta. The CS were probably released over time as indicated by the chromium release studies (10) and the hydrogels eroded slowly as well. This hypothesis cannot be proven until further tests are performed. It is possible the hydrogels were eroded from the time they entered the reticulum and that is how the CS were released. However, this is unlikely as the in vitro studies showed that CS are released over at least 3 days time. Overall, the desired result of release of the CS to stimulate GALT was achieved.

Discussion

Hydrogels were successfully loaded with CS containing a mixture of bacterial antigens including the proteinaceous exotoxin 102 kd in size. Loaded hydrogels released the antigens in the GIT and stimulated an immune response that resulted in protection of the lungs of calves challenged with viable P. haemolytica. It is not clear what factor(s) of the immune system were responsible for the protection. Even though there was no clearly significant increase in pulmonary isotypic antibodies, 3 isotypes did increase in vaccinated calves compared to controls. The lack of significance is due to great variability between calves which are outbred and naturally possess a varied genetic pool. Further statistical analysis to evaluate the immune response is in progress. The pulmonary IgA titer increased the most and approached statistical significance. This is the isotype which is usually seen at mucosal surfaces following stimulation of GALT. Pulmonary IgA could reduce the severity of pneumonia by decreasing the binding of bacteria to epithelial cells in the lung thereby preventing colonization and infection, or by neutralizing the leukotoxin and preventing damage to neutrophils and macrophages in the lung. Release of oxidative radicals by damaged phagocytes can contribute to the damage to the lung parenchyma(12). The role of mucosal immunity in pneumonic pasteurellosis is not well understood at this time. Results of this study suggest mucosal immunity is more important than humoral antibodies which were unchanged in vaccinated calves. Humoral immunoglobulin responses to P. haemolytica vaccines are not necessarily associated with protection(13). The results of this study are consistent with those of previous studies in which intraduodenal administration of CS of P. haemolytica resulted in enhanced pulmonary antibodies in calves(8). The immunity and decreased lesions in vaccinated calves in the present study show that antigen release by hydrogels was as effective as intraduodenal inoculation in stimulating mucosal immunity in calves.

This study showed that hydrogels

can deliver antigens orally to ruminants resulting in immunity at distant mucosal sites. Studies are underway to determine what other antigens can be loaded into hydrogels and retain their immunogenicity when released into the lower GIT. Hydrogels provide a practical, safe, economical way to deliver oral vaccines to a large number of animals to prevent diseases which begin at a mucosal surface.

Acknowlegments

This work was supported by Animal Health and Disease Research Funds at the School of Veterinary Medicine, Purdue University, West Lafayette, IN.

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Summary (English)

Oral vaccination of calves was performed using hydrogel polymers absorbed with culture supernatants of P. haemolytica. Orally vaccinated calves and control non-vaccinates were challenged with virulent P. haemolytica, euthanatized, and pneumonic lesions scored. Vaccinated calves had significantly less pulmonary lesions than nonvaccinates, an increase in pulmonary IgG, IgA, and IgM, and no change in serum antibodies. This study demonstrates that oral vaccination using hydrogels is a useful way of enhancing pulmonary immunity of cattle.

Summary (French)

Un groupe de veaux a été vacciné par voie orale avec le surnageant de cultures de P. haemolytica incorporées dans un polymère hydrogel. Les veaux vaccinés et les veaux témoins ont été inoculés avec des cultures virulentes de P. haemolytica, ont ete sacrifiés, et la sévérité des lésions pulmonaires a été quantifiée. Les lésions pulmonaires des veaux vaccinés étaient moins sévères. Les concentrations d'IgG, IgA et IgM étaient supérieures au niveau du poumon des animaux vaccinés mais semblable dans le sang. Dette étude démontre que la vaccination par voie orale avec des polymères hydrogels est bénéfique pou augmenter l'immunité locale du poumon.

Summary (German)

An Kaelbern wurde eine Schluckimpfung mit Hilfe von Hydrogelen, die mit Kulturueberstaenden von P. haemolytica versetzt waren, durchgefuehrt. Die so geimpften Kaelber und nicht geimpfte Kontrollkaelber wurden mit virulenter P. haemolytica infiziert, eingeschlaefert und die Lungenlaesionen gezaehlt. Geimpfte Kaelber zeigten signifikant weniger Lungenlaesionen als nicht geimpfte Kaelber, einen Anstieg an IgG, IgA, und IgM in der Lunge und keine Veraenderung in Serumantikoerpern. Diese Studie zeigt, dass eine mit Hydrogelen durchgefuehrte Schluckimpfung eine geeignete Methode ist, um die Immunitaet in der Lunge von Rindern zu erhoehen.