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Research Paper

Action of phosphorylated *mannanoligosaccharides* on immune and hematological responses and fecal consistency of dogs experimentally infected with enteropathogenic *Escherichia coli* strains

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Abstract

The therapeutic action of phosphorylated mannanoligosaccharides (MOS) was investigated regarding its prebiotic activity on enteropathogenic *Escherichia coli* (EPEC). Diarrhea was induced in dogs by experimental infection with EPEC strains. Then MOS was supplied once a day, in water for 20 days. Immunological (IgA and IgG), hematological (lymphocytes, neutrophils and monocytes) and bacteriological variables (PCR detection of the *eae* gene of EPEC recovered from stool culture), as well as occurrence of diarrhea were evaluated. All strains caused diarrhea at 24, 48 and 72 h after infection. PCR results indicated that *E. coli* isolated from stool culture of all infected animals had the *eae* gene. There was no significant difference among groups as to number of blood cells in the hemogram and IgA and IgG production. MOS was effective in recovering of EPEC-infected dogs since prebiotic-treated animals recovered more rapidly from infection than untreated ones ($p < 0.05$). This is an important finding since diarrhea causes intense dehydration and nutrient loss. The use of prebiotics for humans and other animals with diarrhea can be an alternative for the treatment and prophylaxis of EPEC infections.

Key words: enteropathogenic *E. coli*, *in vivo* experiment, dogs, phosphorylated 31 *mannanoligosaccharides*, PCR.

Introduction

Phosphorylated mannanoliglaueosaccharides (MOS) act beneficially since they modulate the immune response of animals by presenting the pathogen to Peyer's patches. Pathogens and toxins associated with MOS form a great blend that is easily identified by the immune system. Studies have reported higher production of serum immunoglobulin A (IgA) in dogs, in addition to higher neutrophil activity (Laue and Tucker, 2006). Bio-Mos® (AllTech) is a phosphorylated mannanoligosaccharide extracted from the cell walls of the yeast *Sacharomyces cerevisae*. This complex is rich in mannose and occupies the pathogen binding site, preventing bacteria from binding to mannose receptors in the intestinal epithelium and colonizing the host cells (Collet, 2000).

Escherichia coli is a facultative anaerobic bacterium that inhabits the intestine of humans and animals (Drasar and Hill, 1974). The following diarrheagenic *E. coli* variants are currently known to have acquired virulence: *Shiga toxin*-producing *E. coli* (*STEC*), enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enterohemorrhagic *E. coli* (EHEC) and enteroaggregative *E. coli* (EAEC) (Nataro and Kaper, 1998).

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The lesions produced by EPEC infections in the intestinal epithelium are named attaching and effacing (A/E) lesions. This phenomenon is characterized by intimate bacterial adherence to the intestinal epithelium. The presence of A/E lesions is associated with a disarranging in the enzymatic system of digestive absorption, leading to malabsorption (Nataro and Kaper, 1998) and accumulation of actin and other cytoskeletal proteins, which results in the formation of pedestal-like structures (Cray and Moon, 1995). In the II International Symposium on EPEC, in 1995, researchers classified EPEC samples into two categories (Kaper, 1996): typical EPECs, which cause A/E lesions and have the *eae* gene and the EAF plasmid but do not have the *stx* gene; and atypical EPECs, which cause A/E lesions and have the *eae* gene but do not have the *stx* gene and the EAF plasmid (Nataro and Kaper, 1998). Thus, studies on virulence factors, infection forms and clinical symptoms have been essential to understand the pathogenesis of these bacteria.

Gouffaux *et al.* (2000) evaluated EPEC in humans, dogs and cats by isolating a heterogeneous group of genes using PCR and concluded that at least five genes of these EPEC isolated from animals were closely related to those of human EPECs. This demonstrated the zoonotic potential of dog's EPECs, an important cause of childhood diarrhea in developing countries (Nataro and Kaper, 1998).

The aims of this study were to investigate the therapeutic action of MOS in experimental infection caused by enteropathogenic bacteria in dogs; to evaluate the immune response and the presence of diarrhea; and to propose an *in vivo* EPEC infection model for young dogs.

Materials and Methods

Bacterial strains

Seven EPEC strains were used, four atypical (*eae*+) and three typical (*eae*+, *bfpA*+) strains (Beaudry *et al.*, 1996; Nakazato, *et al.*, 2004), as shown in Table 1. All samples were negative for the Shiga toxin gene (*stx*) (Blanco *et al.*, 1997). Adherence characteristics of the strains used in the present study are shown in Table 1.

Animals

Twenty-five male and female Boxer dogs aged 60 days were used. The animals were selected from five litters of four sisters aged one and a half year. All animals were from the same breeder.

The parents were vaccinated (Vaccine Duramune® Max (Fort Dodge)) when they were pups and regularly dewormed (Vermifuge Endal Plus® (10 mg/kg)) at every four months. Pups were dewormed with Praziquantel and Pyrantel Pamoate when aged 20 days and vaccinated against distemper, infectious hepatitis, adenovirus type 2, parainfluenza, parvovirus and coronavirose when aged 45 days.

Table 1 - Characteristics of dog EPEC strains used in the experimental infection *in vivo* (1,20).

Strains	Presence of			Adherence to HEp-2 cells
	eae[*]	*bfpA*[•]	EAF[#]	
008	+	-	-	LAL[¥]
HE8	+	-	-	-
SPA14	+	-	-	LAL
SPA16	+	-	-	NC
4225	+	+	+	-
4083	+	+	+	-
3549	+	+	+	-

[*]eae: gene that codes for intimin.
[•]*bfpA*: one of the genes responsible for codifying bundle-forming pilus.
[#]EAF: EAF plasmid detection.
[¥]LAL: localized adherence-like pattern.
NC: *non-characteristic adherence* pattern.
+: positive.
-: negative.

Clinical examination and complete blood count were performed for the parents of experimental animals in order to evaluate their health status during mating. All pups were subjected to clinical evaluation at birth, at 20 days and 45 days after birth and before the beginning of the experimental period.

Facilities

After mating, females were kept in individual masonry kennels with covered area and solarium. From birth to weaning, at 30 days after birth, pups remained close to their mother in the same kennel. During the whole pregnancy and breastfeeding period, the mothers received animal food (AGR® Royal Canin) and clean and fresh water *ad libitum*. During weaning, the pups of each litter were housed in kennels, where 3 pups of one same litter were kept in one masonry kennel which also had covered area and solarium; pups received the same diet provided to their mothers.

Experimental infection

The spread of *E. coli* strains was performed in BHI for 24 h at 37 °C without agitation. This bacterial growth was centrifuged and resuspended in saline (0.85% NaCl) until reaching Mcfarland scale 8 (2.4 x10^9 bacteria/mL). The quantity of bacteria was verified by counting the colony forming units (cfu) in McConkey agar (Sigma). The animals were experimentally infected with 1 mL of resuspended bacteria by the oral route using gelatin capsules of intestinal release at a single dose. After 48 h of experimental infection, all animals presented liquid-to-pasty diarrhea without vomit. Feces were collected by using a sterile swab and inoculated into GN broth incubated at 37 °C overnight for 24 h, which was then sown on McConkey agar (Sigma) incubated at 37 °C for 24 h; one sample per animal was

used. One colony from each growth was selected, inoculated into Brain Heart 1nfusion (BHI – Oxoid) broth and incubated at 37 °C overnight for genomic DNA extraction, as described by Gouveia *et al.* (2011).

PCR

For PCR of the *eae* gene, 50 ng DNA template were added to 2.5 U *Taq* DNA polymerase (Invitrogen), 50 pmol of each primer, 200 mM deoxynucleoside triphosphate (Invitrogen), 1.5 mM $MgCl_2$ (Invitrogen) and 1X PCR buffer (Invitrogen) at 25 mL final volume (Nakazato *et al.*, 2001).

After an initial denaturation at 94 °C for three minutes, samples was subjected to 35 thermal cycles at 94 °C (denaturation) for one minute, 56 °C (annealing) for one min and 72 °C for 40s. Reactions were performed in a thermocycler BioRad Laboratories, USA. A 5 mL volume of each reaction was subjected to 0.8% agarose gel electrophoresis, stained with ethidium bromide or SYBR Gold (Invitrogen) and visualized in a transilluminator (Ultra Violet Products). Amplification of an 815 bp fragment was expected (Nakazato *et al.*, 2001).

For amplification of the *eae* gene, the following primers were used (Gannon *et al.*, 1993):

EAE1: 5'ACGTTGCAGCATGGGTAACTC3' and EAE2: 5'GATCGGCAACAGTTTCACCTG3'

Experimental design

Five experimental groups were defined: Group A - no diarrhea induction and no MOS supply; Group B - diarrhea induction by the strain 4083 and no MOS supply; Group C - diarrhea induction by the strain SPA14 and no MOS supply; Group D - diarrhea induction by the strain 4083 and MOS supply; Group E - diarrhea induction by the strain SPA14 and MOS supply

MOS was orally administered at 2 g/kg live weight diluted in 2 mL water once a day during 20 days from 24 h after experimental infection. The inoculation day was considered the Day zero of the experiment.

Experimental design and statistical analyses followed the instructions of Kaps and Lamberson (2004). Experimental design was completely randomized with five replicates, and the effect of groups was analyzed at each moment. To evaluate the effect of moments for each experimental group, a randomized complete block design was adopted, considering the animal a blocked factor.

Response variables

All animals were clinically evaluated according to Jones (2003) at every six hours after experimental infection in the first five days. After this period, examinations were performed at every 24 h.

Clinical evaluation

In all five moments of the experimental period (days 0, 5, 10, 15 and 20), the animals were also clinically evaluated through measurement of body temperature and assessment of dehydration degree (mild, moderate and intense), mucosal color (pale, pink and congested), vitality (apathetic, plays when stimulated and agitated), presence of blood in the feces (absent, little and much blood) and fecal consistency (normal, pasty and liquid).

Laboratory evaluation

Soon after clinical evaluation, a 5 mL blood sample was collected from each animal by jugular puncture. These samples were separated into two aliquots. The first aliquot was conserved in a sterile flask with anticoagulants (EDTA) at -2 to 8 °C for subsequent hemogram using an ABC Vet automated analyzer. The second aliquot was centrifuged and, after separation, the serum was kept at -2 to 8 °C for subsequent determination of total IgA and IgG levels by ELISA (using the commercial kits IgA ELISA quantitation kit and Dog IgG ELISA quantitation set, Bethyl Laboratories). Of the samples obtained with the second aliquot, only those of days 0, 10 and 20 after inoculation were used. After collection of each blood sample, the animals were individually weighed.

Statistical analysis

The results of hemogram, immunoglobulin dosages and fecal consistency of groups were compared at each moment by the Kruskal-Wallis test, while these responses at the different moments were compared for each group by the Friedman test, according to Kaps and Lamberson (2004). For all comparisons, significance level was set at 5% ($a = 0.05$).

Ethics committee

This study was duly approved by the *Animal Experimentation Ethics Committee*/Federal University of Mato Grosso do Sul (UFMS), protocol number: 116/2006. After the experiment, all animals had good health status.

Results

PCR

At the beginning of the experiment, seven EPEC strains were evaluated, 3549, 4083, 4025, 008, HE8, SPA14 and SPA16. All strains were tested by PCR and showed amplification of an 815 bp fragment, corresponding to the *eae* gene.

Hemogram

The medians of white blood cell concentrations at different days post infection (dpi) for animals of all experimental groups were calculated. There was no significant differences ($p > 0.05$) among groups at the several dpi and among dpi for the several groups. Before the experiment, hemogram and hemoparasite survey were performed and

all animals were within the normal parameters for blood cells and had negative results for hemoparasite survey.

Immunoglobulins IgG and IgA

The medians of IgA and IgG levels at different dpi for animals of all experimental groups were also calculated. There was no significant differences ($p > 0.05$) among groups at the several dpi and among dpi for the several groups.

Clinical evaluation

The body temperature of all animals remained within the normal limits, ranging from 38.5 °C to 39 °C. No animal vomited or had dehydration symptoms, indicating absence of parenteral hydration over the experiment. Mucosal color remained normal and the animals were alert (not apathetic). Blood was not detected in the feces at any moment. Amplification of the *eae* gene (815 bp) by PCR led to identification of EPEC SPA14 and 4083 strains in the feces of all experimentally infected animals.

Results of fecal consistency for the different experimental groups and at different dpi are shown in Table 2.

All animals inoculated with EPECs had pasty diarrhea for 12-24 h, including the treated groups D and E. No control animal had symptomatology of intestinal infection, whereas animals of the remaining groups had intense diarrhea, presenting liquid feces at a certain moment over the experimental period. Bio-Mos®-treated animals (Groups D and E) showed a significantly ($p < 0.05$) faster recovery relative to the animals that did not receive the prebiotic (Groups B and C). At fifteen days after treatments, all animals had already recovered.

Discussion

The action of MOS is to bind to type-1 mannose site in the bacterium, preventing the latter from binding to glycoproteins of intestinal cells. According to Ferket (2004), mannanoligosaccharides may stimulate the immune response against specific pathogens, preventing their colonization and promoting their presentation to the immune system as attenuated antigens. On account of such binding capacity with enteric pathogens (Spring *et al.*, 2000), the process of activation by antigens of blood cells (neutrophils, lymphocytes and monocytes) would be impaired since the bacterium would leave the intestine without causing damage to the host. This may be the reason why cell immunity was not activated to a sufficient level. It must be emphasized that the type-I fimbria receptor (most common among *E. coli*) has D-mannose as receptor (the same as that of MOS); thus, the most probable mechanism of EPEC inhibition would be by competition of adhesion receptors. However, the hypothesis of immunoglobulin stimulation should not be ruled out.

Table 2 - Fecal consistency at five days of the experimental period for dogs subjected to different treatments*.

Groups	Time points (days)				
	0	5	10	15	20
A	normal	normalpasty diarrhea	normal	normal	normal
B	normal	pasty diarrhea	pasty diarrhea	normal	normal
C	normal	pasty diarrhea	pasty diarrhea	normal	normal
D	normal	pasty diarrhea	normal	normal	normal
E	normal	normalpasty diarrhea	normal	normal	normal

*A - No diarrhea induction and no MOS supply; B - Diarrhea induction by the strain 4083 and no MOS supply; C - Diarrhea induction by the strain SPA14 and no MOS supply; D - Diarrhea induction by the strain 4083 and MOS supply; E - Diarrhea induction by the strain SPA14 and MOS supply.

There are few studies about the effect of prebiotics on the blood parameters of the several species, and those for the canine species are even scarcer. A situation similar to that of the present study was reported by Budiño *et al.* (2004) who carried out an experiment to test the addition of prebiotics for weaned piglets and did not find differences between treatments for haematological variables.

Laue and Tucker (2006) stated that pathogens and toxins associated with MOS form a great blend easily identified by the immune system. In our study, there was no change in serum IgA and IgG levels. Thus, future studies should quantify IgA from samples of the intestinal mucosa, which can be more easily stimulated by the blend formed by the association between MOS and pathogens/toxins.

The mucosa surfaces of the intestine develop active defense mechanisms mediated by cells and chemical factors, both related to innate (nonspecific) and acquired (specific) immunity. These systems show differences that, although not marked, are due to the different external factors to which they are subjected (Silva, 2006). For pups, these external factors, such as stress, cold and heat, are difficult to control, which influences the levels of immunoglobulins. This could be minimized if the number of animals were larger, as well as the number of replicates. In the present study, samples were from pups from sister mothers and the difficulty in carrying out experiments with a large number of specimens limited the number of samples per treatment.

Based on virulence markers associated with diarrhea in humans and animals, five diarrheagenic groups of *E. coli* were defined (Beutin, 1999), including the enteropathogenic group (EPECs), expressing colonization factors such as intimin (codified by the *eae* gene) and bundle-forming pili (BFP).

In the present study, typical (strain 4083) and atypical (strain SPA14) EPEC strains caused diarrhea in the animals at 24, 48 and 72 h after induction, suggesting that both strains were pathogenic for the tested dogs. There was no difference between the results of typical and atypical sam-

ples, which leads us to believe that the BFP fimbria was not essential for diarrhea manifestation; thus, BFP was not deeply investigated in the present study.

EPECs have virulence factors associated with several intestinal diseases in humans (Levine, 1984) and other animals (Pestana de Castro *et al.*, 1984; Francis *et al.*, 1991; Blanco *et al.*, 1993). Since the dog is a domestic animal which lives closely with humans, the symptoms of natural infection caused by the studied strains indicate that animals could potentially contaminate humans and vice versa.

Nakazato *et al.* (2004) reported that the EPEC serotypes found in humans were also identified in other animals including dogs, demonstrating the zoonotic risk of EPEC from dogs. All *E. coli* samples isolated from the feces of inoculated animals had the *eae* gene, *i.e.* 100% of the isolated colony-forming units were EPECs, evidencing intense bacterial colonization of the intestinal epithelium, which indicates that dogs can represent an important source of EPEC infection for humans.

MOS had an effect on fecal consistency, and MOS-treated animals recovered more rapidly from diarrhea than infected animals not treated with MOS. By means of competitive inhibition by mannose receptors, MOS decreases the effect of bacterial adherence by type-I fimbria in the intestinal epithelium of animals, an important process involved in EPEC pathogenicity. Since type-I fimbria is found in most *Escherichia coli* samples (Law, 1994), the presence of MOS may potentially have reduced the colonization or the perpetuation of colonization. Furthermore, the evaluation of fecal consistency (presence of diarrhea) showed that the process of bacterial adherence is extremely important in the emergence of diarrhea in EPEC-infected dogs.

In the present study, the EPEC infection model in dogs was established to determine the pathogenicity symptoms of these samples. Dogs infected with EPEC receiving MOS demonstrated faster remission of diarrhea.

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