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Preliminary Study: Evaluation of Glyceryl Monooleate Cubic Phase as a Protection and Carrier System for *Actinobacillus pleuropneumoniae* Toxins in Mice

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Abstract: In the present research, the capacity of the cubic phase of glyceryl monooleate was studied as a carrier and protection vehicle for Apx toxins I, II and III with the aim of obtaining an orally administered immunogen for the protection of susceptible pigs from *Actinobacillus pleuropneumoniae* infection. In the presence of high water concentrations and at body temperature, the monoolein, an amphiphilic lipid, produces what is called the cubic phase which is characterized by the formation of a curved double layer which extends to three dimensions, thus separating two congruent water channel systems that form a pore of approximately 5 nm in which the Apx toxins were trapped. Apx toxins I, II and III were obtained from the supernatants of cultures of *A. pleuropneumoniae* serotypes 1, 3, 5 and 7. The capacity of the monoolein gel to trap the apx toxins as well as the effect of their inclusion in the cubic phase were evaluated by polarized light microscopy. The gel toxin release was evaluated using *in vitro* dissolution tests. The monoolein gel was able to retain a 400 μg mL^{-1} antigen concentration without affecting the formation of the cubic phase. Approximately 60% of the protein molecules were released from the gel within 4 h. The antigenic effect of the product, orally inoculated in mice was observed in *in vivo* studies with changes in the T-lymphocyte and B-lymphocyte populations being identified by flow cytometry and antibodies against the antigen were sought using the ELISA test. Small changes were detected through flow cytometry in lymphocyte populations with the CD4 and CD19 markers allowing to speculate on the possible stimulation of the immune system by the antigen, which was confirmed by the appearance of antibodies in the animals' serum, identified by the ELISA test. The results obtained are encouraging and open the possibility of developing an orally administered immunogen to protect pigs from becoming infected with *A. pleuropneumoniae*.

Key words: Cubic phase, monoolein, apx toxins, *A. pleuropneumoniae*, porcine plueropneumonia, oral vaccine

INTRODUCTION

Porcine pleuropneumonia is a highly contagious disease which is particularly associated with growing pigs, it possesses an extraordinary economical interest due to the high death percentages and even more important, the decrease in production, the lengthening of the fattening period, the high medication costs and veterinary assistance (Mendoza and Ciprian, 2001).

The disease may vary from a hyperacute to a chronic course. The characteristic acute lesion is a necrotic hemorrhagic pneumonia associated with fibrinous pleuritis, while the chronic lesion is characterized by a consolidated, infarcted and encapsulated lung tissue. The microorganism is transmitted through sprays or by direct contact being able to cause the animals death at any age and those who survive become chronic carriers (Hensel *et al*., 1995). The etiologic agent is the *Actinobacillus pleuropneumoniae* bacterium. This microorganism possesses several pathogenicity factors, which interact among themselves to produce pleuropneumonia in susceptible pigs (Liao *et al*., 2003).

These factors include: the capsule, the lipopolysaccharide, the cytoadherent fimbriae, the iron uptake systems and the production of exotoxins called Apx toxins, which show hemolytic and cytotoxic activity. Although, it is known that the *Actinobacillus pleuropneumoniae* virulence is multifactorial, several studies point out that its virulence is strongly related with the production of toxins. There are four types of Apx toxins. Type 1 which is strongly hemolytic and non-cytoxic and is produced by serotypes 1, 5, 9, 10 and 11.

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Type 2 is slightly hemolytic and cytotoxic and it is produced by almost all serotypes (1-9, 11 and 12). Type 3 is non-hemolytic but highly cytotoxic and it is produced by serotypes 2, 3, 4, 6 and 8 and Type 4, which has been recently identified is only produced *in vivo* by all serotypes. In porcine pleuropneumonia, the toxins' activity is probably responsible for the initial injures, which are characterized by being hemorrhagic and necrotic. The vaccines initially manufactured against pleuropneumonia frequently failed in trying to confer a sufficient protection against the disease, besides of being useful only for the specific serotype for which they were prepared.

Nowadays, there are other kind of vaccines available in the market, which combine bacterines and toxoids that have been used with better results; however, these still use the intramuscular and subcutaneous administration route. It is well known that the immunogen administration route has an influence on how and where an immune reaction occurs. The concept of an integral mucosal immune system has already been established by several studies using the oral administration route. The development of orally administered immunization methods may be of great importance for the control of this disease (Hensel *et al*., 1995).

Glyceryl monooleate, a molecule used since the 1950s as an emulsifier and additive in some aliments has raised great interest in the pharmaceutical industry in recent years due to its low toxicity, biodegradability and biocompatibility features. It is an amphiphilic lipid that upon contact with water, forms transition phases which result in the existence of intermediate stages called liquid crystal phases, which depend on the lipid's structural properties, the water content and temperature (Chang and Bodmeier, 1997a; Engstrom *et al*., 1992). In the presence of an excess of water and at body temperature, the monoolein forms a viscous gel known as cubic phase, which molecularly consists of a curved double layer which extends to three dimensions, thus separating two congruent water channel systems that form an aqueous pore of approximately 5 nm (Ganem-Quintanar *et al*., 2000). These characteristics allow the monoolein to research as a carrier and protection system for protein molecules (Chang and Bodmeier, 1997b; Ericksson *et al*., 1983).

The mechanism for the release of the protein molecules trapped in the cubic phase channel system is the dysfunctional exchange of water from the external environment with the matrix with the consequent exchange of water and protein molecules from the inside to the outside medium (Longer *et al*., 1996). The objective of this study was to evaluate the capacity of the glyceryl monooleate cubic phase to protect and carry orally administered Apx toxins to the immunologic recognition sites in the animal's intestine, where an immune reaction with potentially protective characteristics against the disease may occur.

MATERIALS AND METHODS

Bacterial strains and seed obtainment: The supernatants were obtained from a 12 h growth in heart infusion broth supplemented with 20% yeast extract of serotypes 1, 3, 5 and 7 from *Actinobacillus pleuropneumoniae* field strains, which were inactivated with formaldehyde to a final concentration of 0.5%. The supernatants were concentrated by tangential filtration and lyophilization.

Characterization of apx toxins: The Bradford method was used for the determination of protein concentration in order to indirectly evaluate the amount of toxins present in the supernatants. The SDS-PAGE analysis was used to establish the presence of the toxins in the supernatants (Bradford, 1976).

Antigen dosage: In order to determine the amount of antigen that was used to immunize the animals, cytotoxicity assays were carried out in VERO and PK-15 cultured cells as well as hemolytic activity tests on sheep erythrocytes. A microplate was prepared for each type of cell line until a confluence of 90% in the RPMI medium (ICN Biomedical Inc., USA) was achieved, which were inoculated with 100 μL of different protein concentrations (100, 200, 300, 400, 500 and 600 μg mL^{-1}) of each bacterial serotype. The hemolytic activity of the *Actinobacillus pleuropneumoniae* supernatants was determined using sheep erythrocytes in TS buffer (tris 10 mM NaCl 0.8%, pH 7.5). About 1 mL of filtered supernatants with an initial concentration of 1000 μg mL^{-1}, or dilutions of these in TS buffer at 500, 250, 125, 62.5, 31.2, 15.6, 7.8 and 3.9 μg mL^{-1} concentrations were mixed with 1 mL of 1% sheep erythrocytes. $CaCl_2$ was added to a final concentration of 100 mM. It was incubated for 2 h at 37°C, the non-lysated erythrocytes were removed after centrifugation at 2500 rpm for 5 min and the amount of hemolysis was finally measured by determining the absorbance of the samples at 540 nm (Bagdasarian *et al*., 1999).

Monoolein gel evaluation: The monoolein cubic phase formation and the effect of toxin inclusion in the gel was determined by polarized light microscopy studies as well as by the visual observation of the system formed; the antigen release was determined using *in vitro* dissolution tests. Briefly, an aqueous solution containing 200 μg of

Apx toxins in a volume of 300 μL was added to 700 mg of monoolein and was centrifugated at 14000 rpm for 10 min. Afterwards, the mixture was allowed to balance at room temperature for 24 h.

The toxins' release rate was measured in *in vitro* dissolution tests and the protein content was determined by the Bradford method. Initially, the type of membrane that could be used in the assay was identified, so as not to affect the release from the monoolein by the control of passage through the membrane used (Whatman filter paper). A sample of monoolein gel cubic phase, containing 200 μg of Apx toxins antigen was tested. Samples were taken at 30, 60, 90 and 120 min and at 4, 8 and 24 h, measuring the concentration of proteins released in the dissolution mixture.

Animals: About 20 male CD1 mice of approximately 15 g from a pathogen-free bioterium were used. They were distributed in cages with five animals each, except for the experimental group for which mice were placed in individual cages. Mice were fed once daily with regular food and were given water *ad libitum*.

Experimental design: A group of 5 animals was used as negative control to which only physiologic saline solution mixed with food was administered. As positive control, 5 mice were immunized with the Ingelvac Appx vaccine by intramuscular route (bacterine-toxoid against *Actinobacillus pleuropneumoniae*; 1, 2, 3, 4, 5, 7 serotype culture; chemically inactivated apx I, II, III toxoid; aluminum hydroxide as adjuvant). Inoculation was performed on days 0, 7 and 14. A group of 5 animals received the monoolein gel without the antigen in order to establish whether the vehicle affects the result in any way. The monoolein cubic phase without the antigen was applied on days 0, 7 and 14 of the assay. Monoolein cubic phase containing 400 μg of the antigen mixed with food was applied to the experimental group. The animals were kept in individual cages to make sure they received the correct doses. The orally taken antigen was applied on days 0, 7 and 14 of the assay. All animals were euthanized by day 28 and blood and tissue samples were taken from each animal.

Flow cytometry: The flow cytometry assay (Cyflow space-partec VASTELL, USA) was used to prove the changes in the lymphocyte population presenting the CD3, CD4, CD8 and CD19 markers as a way of evaluating the cellular and humoral response. The tissue used was the spleen from which a cell suspension containing between 200 and 250,000 cells mL^{-1} was obtained. Once the tissue was removed, it was passed through a mesh in order to obtain individual cells, which were washed 3 times with physiologic saline solution and were subsequently resuspended in RPMI broth. In order to adjust the number of cells, these were counted in a Neubauer chamber using a vital dye (Trypan blue). About 30 mL of each sample were incubated for 30 min with 5 μL of the labelled monoclonal antibody. Then, 200 μL of lysis solution were added, it was shaken and incubated for 10 min under darkness conditions. Finally, three washings were carried out using physiologic saline solution, which was discarded after the last washing, resuspending the cell pack in 500 μL of 4% paraformaldehyde, keeping the suspension under refrigeration until its reading in the flow cytometer.

ELISA test: An indirect ELISA test was performed to identify antibodies against the antigen in the animals' sera. For antigen pasting, this was diluted to a concentration of 10 μg mL^{-1} using a 0.1M pH 9.5 carbonate buffer solution. About 100 μL of this solution were placed in each well of the ELISA plate with overnight incubation at 4°C. Afterwards, this was washed 3 times with 300 μL of a 0.05% PBS/Tween 20 solution and was blocked for 1 h with 5% skim-milk. After washing, the test sera diluted 1:10 were added and were incubated for 2 h then, they were washed with PBS/Tween 20 buffer solution and 100 μL of peroxidated A protein were added. It was incubated for 1 h at room temperature. After washing, the OPD enzyme substrate (ortho-phenylendiamine citrate + hydrogen peroxide, SIGMA Fast opd, USA) was added at room temperature. The reaction was stopped using 50 μL of 4 N sulfuric acid. Finally, the absorbance of each well was read at a 492 nm wave length.

RESULTS AND DISCUSSION

The material obtained after lyophilization was resuspended in 10 mL of phosphate buffer solution and was kept under freezing at -20°C until the performance of tests. Dilutions of bovine serum albumin were made, containing between 1 and 10 μg of protein for the preparation of the calibrating curve that was used to quantify the protein concentration obtained from the supernatants in the 1, 3, 5 and 7 serotype broths of *A. pleuropneumoniae*.

In order to establish a relationship between the variables tested, a graph was prepared and the correlation coefficient was obtained. The correlation coefficient (r^2 = 0.996) causes the results obtained by using this curve to be reliable. With the data and the graph obtained, it was possible to establish the protein

Table 1: Protein concentration obtained from the supernatants of the samples of App 1, 3, 5 and 7

Supernatant	Protein concentration (mg mL^{-1})
App 1	3.40
App 3	2.92
App 5	3.77
App 7	3.31

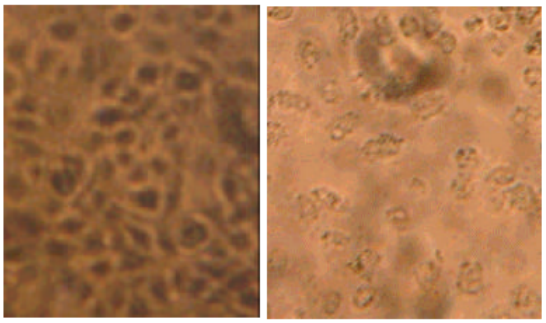

Fig. 1: Normal PK-15 cultured cells (left). Flattening and rounding of PK-15 cells by the Apx toxins (right)

concentration in the supernatants of the samples of App 1, 3, 5 and 7; the results are shown in Table 1. These data allow to have an approximate estimation of the amount of Apx toxins present in the supernatants, since not all of the proteins quantified in the system were toxins. About 10 μL of each supernatant were used to carry out the electrophoresis in 10% SDS polyacrylamide gel. By using a molecular weight marker (Sigma Marker High Weight, USA), it was possible to identify 100 kDa strips close to the marker for all of the App supernatants tested. Taking into account that the molecular weight of the toxins is 105 for Apx I, 103 for Apx II and 120 for Apx III, the results obtained suggest the existence of the Apx toxins in the supernatants.

In order to titrate the amount of protein used as antigen, 2 assays were carried out. The first assay evaluated the cytotoxic effect of the toxin on cultured cells; the cytopathic effect observed was the flattening and rounding of the cells. The wells showing this effect in at least 50% of the monolayer were considered as positive. In Fig. 1, the effect produced by the toxins on PK-15 cultured cells is observed.

The second assay evaluated the antigen's hemolytic capacity. From the results obtained in both assays, the decision was made to use a 400 μg antigen concentration as immunogen. Once the amount of antigen to be used as immunogen was determined, the effect of the antigen's inclusion in the formation of the glyceryl monooleate cubic phase was tested to do this, a polarized light microscopy was used, since the monolein cubic phase is isotropic (it should not deviate the polarized light). By using a system of polarized glasses, it was established

Table 2: Protein concentration in the dissolution medium at different times

Time (H)	Protein concentration (μg mL^{-1})
0.50	0.24
1.00	1.00
1.50	2.94
2.00	4.56
4.00	6.34
8.00	6.41
24.00	6.37

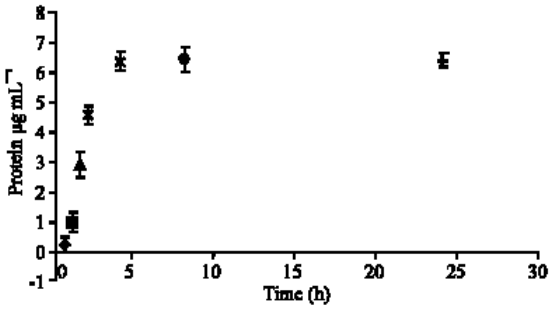

Fig. 2: Graphic of the *in vitro* dissolution tests

that the inclusion of 400 μg of Apx toxins' antigen would not interfere with the formation of the cubic phase. Once the App toxins were trapped in the water pores of the monoolein cubic phase, it was necessary to determine if the antigen would be released later on.

For this purpose, *in vitro* dissolution tests were used. For the performance of these tests, dissolution cells were used, since they allow to separate the solution sample from the dissolution by means of a membrane. Initially, assays were carried out in order to find a membrane that would not interfere with the tests' results (Table 2). This membrane was Whatman paper N°5. The results of the *in vitro* dissolution tests are shown in Fig. 2.

Evaluation of the experimental model: The measurement of the percentages and the standard deviation for each labelled lymphocyte population belonging to each of the groups tested are shown in Table 3. The results graphs shown in Fig. 3-6 allow to observe very small variations among the different T- and B-lymphocyte groups, which may be interpreted as little animal reaction to the orally taken antigen stimulation.

Through, the indirect ELISA test, the presence of antibodies against the antigen, prepared with apx toxins I, II, III was observed. The mean and standard deviations of the results obtained are shown in Table 4, indicating the absorbance measured at 492 nm.

Porcine pleuropneumonia has a great economic importance world-wide; therefore, the development of more effective control methods for this disease is required. Since this is a pathogen which causes mucosal infection, the stimulation of an immune response at this

Table 3: Percentages and the standard deviation for each labeled lymphocyte population belonging to each of the groups tested

Groups	CD 3	CD 4	CD 8	CD 19
(-) Control	36.6/3.3	16.94/1.48	7.96/1.93	34.32/3.99
Gel control	36.6/2.32	16.74/1.54	7.47/0.66	35.50/3.43
(+) Control	36.02/2.83	16.64/2.89	7.62/0.52	35.72/2.39
Oral vaccine	38.02/2.11	17.52/0.63	7.44/0.86	37.66/2.11

Table 4: Mean and standard deviations of the absorbances measured at 492 nm obtained from an indirect ELISA test of the serum animals

Groups	Absorbance (492 nm)	SD
(-) Control	0.046	0.002
Gel control	0.050	0.003
(+) Control	0.345	0.018
Oral vaccine	0.350	0.013

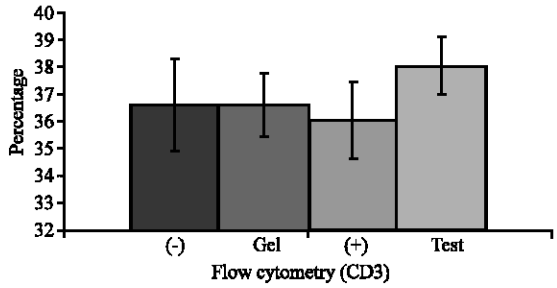

Fig. 3: Percentages and the standard deviation of the cells with the CD3 marker from each experimental group obtained by flow cytometry

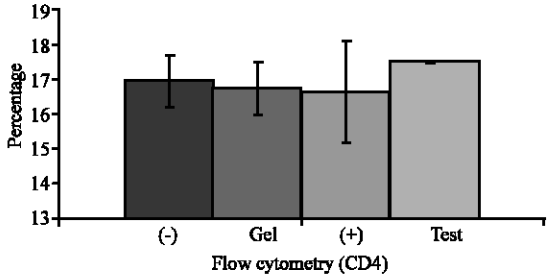

Fig. 4: Percentages and the standard deviation of the cells with the CD4 marker from each experimental group obtained by flow cytometry

level may confer a better protection against this disease. The use of an orally administered immunogen requires a carrier and protection system for the antigen; these systems are usually based on the use of polymeric carriers.

A great attention has been given to lipids as alternatives to the use of polymeric carriers or covering materials in the development of drug transport and protection systems.

The major advantages over polymers include on the one hand, the low melting temperature, which avoids the usage of organic solvents and the consequent absence of impurities, such as residual monomers and catalysts and

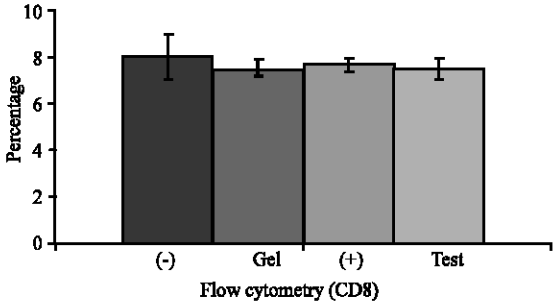

Fig. 5: Percentages and the standard deviation of the cells with the CD8 marker from each experimental group obtained by flow cytometry

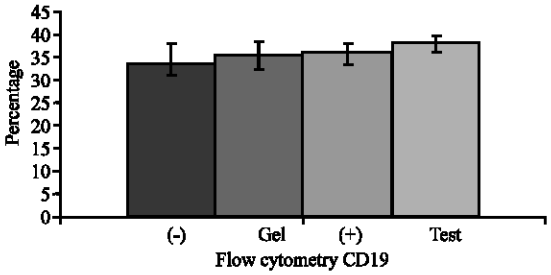

Fig. 6: Percentages and the standard deviation of the cells with the CD19 marker from each experimental group obtained by flow cytometry

on the other hand, its potential biocompatibility and biodegradability (Chang and Bodmeier, 1997a, b). Glyceryl monooleate, an amphiphilic lipid that has recently raised great interest in the pharmaceutical industry due to its low toxicity and biodegradability properties may work as a carrier and protection system for protein molecules by using the water channels which are formed when the cubic phase of monoolein is produced at high water concentrations. A protein carrier and protective drug, administered by oral route would allow to take the antigen molecules to the animal's immunologic recognition sites working as an oral vaccine. In this study, an immunogen was developed using *Actinobacillus pleuropneumoniae* Apx toxins I, II and III and the monoolein cubic phase as a carrier and protection system. According to Ericksson *et al*., 1983, the glyceryl monooleate cubic phase may be identified at first sight, since it is completely transparent and very thick, which is consistent with the results observed in this study. These same authors postulate that a more accurate characterization of the glyceryl monooleate cubic phase formation is obtained through polarized light microscopy. It is known that based on its optic behavior, the crystalline substances can be grouped in two categories: anisotropic crystals, those which present different refraction indexes in various crystal-

graphic directions and isotropic crystals, those which present the same refraction index in all directions. The monoolein cubic phase is isotropic, since it does not deviate the polarized light, which was proven in this study when placing a sample of the gel containing 400 μg of Apx toxins between two polarized glasses, where the second glass stopped the beam polarized by the first glass, leading to the observation of a visual field under complete darkness conditions in the polarized light microscope; the visual field under complete darkness conditions confirms that the inclusion of toxins in the system at the concentration tested does not interfere with the formation of its cubic phase, thus being able to work as a carrier and protection system.

Later on, this system should be able to release the antigen. Longer *et al*. (1996) indicate that when in contact with the water environment, the monoolein is swelled and becomes a gel that releases a dissolved and dispersed drug in it by simple and low diffusion. This was proved by the *in vitro* dissolution tests. The results In Fig. 2 shows the protein concentration in the dissolution medium at different times. It can be observed that the protein concentration in the dissolution medium increased over time, which means that the antigen would be released from the gel in which it is contained.

Protein concentration remained stable approximately since the 4th h, which indicates that by that time, the greatest amount possible of the antigen was released, representing nearly 70% of the total antigen, which is consistent with the results of Longer *et al*. (1996), who obtained a very similar percentage and release time in their drug release studies AG337 other researchers, such as Chang and Bodmeier (1997b) found that the drugs included in the glyceryl monooleate cubic phase were constantly released until reaching a 60% release percentage and no greater release of the drug was obtained thereafter, even if the dissolution media conditions were changed. The researchers state this is due to the fact that some molecules of this drug may bind to monoolein preventing its dissolution.

Nevertheless, it is important to mention that the test device in this study did not have the ability to shake the gel allowing its complete dissolution, a situation that is expected under *in vivo* conditions, where it is assumed that monoolein will disintegrate by the action of intestinal peristalsis.

Orally administrated antigens have been shown to effectively induce IgA and IgG antibodies (Challacombe *et al*., 1992). It was argued that the uptake of the antigen by Peyers patches is a pre-requisite for the induction of a substantial immune response (McClean *et al*., 1998; Smith *et al*., 1995). Besides their role in the delivery of the antigen into the Peyers patches, the monoolein cubic phase also protects the active entity from the destructive action of enzymes and the low pH environment of the stomach. In order to evaluate the immune cellular and humoral response of mice to the antigen tested, flow cytometry and the ELISA test were used.

The markers used in flow cytometry were CD3, CD4, CD8 and CD19 antibodies. it is important to point out that although the minimum differences observed in the T- and B-lymphocyte populations in the different groups of tested animals in the experiment are not statistically significant under the one-factor Analysis Of Variance (ANOVA), they may indicate that the oral vaccine somehow causes an immune reaction against it but a more complete study is required to prove its antigenic capacity more accurately.

The administration route of an immunogen has an influence on how and where an immune response occurs. The concept of an integral mucosal immune system has already been established by several studies using the oral administration route (Hensel *et al*., 1995; Felder *et al*., 2000). Some reseachers, such as Hensel *et al*. (1995) have shown the production of high antibody titers in the serum of the animals (pigs) tested using orally administered inactivated bacteria.

This leads to believe that oral immunization may provide a good protection against the disease. The results in Table 4 show that the serum of the positive control groups and the oral vaccine show antibodies against the tested antigen. The identification of antibodies against the antigen in the animals serum, immunized with the oral vaccine indicates that it was able to induce an immune response proving the antigenic capacity of the product. On the other hand, Liao *et al*. (2003) using a microsphere system which protected and carried *A. pleuroneumoniae* cells inactivated with formaldehyde, showed the effectiveness of orally administered immunization systems to prevent *A. pleuropneumoniae* infection. The transport and protection of orally administered protein antigens is of great importance.

CONCLUSION

Based on the tests performed, the glyceryl monooleate cubic phase showed to be a very good carrier and protective vehicle for Apx toxins, able to trap protein molecules in its water channels system; although the results are not statistically significant, they are encouraging, since it was possible to identify some changes in the immune system's cell populations, which may indicate the emergence of an immune response

against the antigen used. The identification of antibodies against the Apx toxins in the serum of orally immunized animals confirms this observation and opens the opportunity for a more detailed study of this methodology.

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