

## Immunization Intraductal Fibronectin and Whole Cells of *Staphylococcus aureus*, *Escherichia coli* and *Streptococcus agalactiae*, in Cows to Dry in Puebla-Mexico

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Nine dairy cows seven months gestation received via the milk ducts formulation inactivated antigen protein adhesion to fibronectin of *Staphylococcus aureus*, *Streptococcus agalactiae* and *Escherichia coli* cells. The analysis of the immunoglobulins G by indirect ELISA against the applied antigens, showed that five of nine cows were stimulated with *Escherichia coli*, the case for the antigenic stimulus for *Streptococcus agalactiae* showed that eight of nine cows responded with good indices of significance. Finally, stimulus virulence proteins of *Staphylococcus aureus* adhesion to fibronectin, revealed response rates all cows (P < 0.0002). Intraductal response to antigens promises to be a way for immunization to dairy cows in the drying stage.

**Keywords:** Acute mastitis, inactivated antigens and analysis of immunoglobulins.

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In order for the mammary gland to become infected with some bacteria, a physical contact between the pathogen and the host is necessary; Where the recognition receptors are exhibited between them, at that time, the innate immune system of the host responds through the interaction of molecules referred to as Pathogen Associated Molecular Patterns (PAMPs) [Ulevitch *et al.*, 2000]. Mastitis cases by Gram negative bacteria are more common, being *Escherichia Coli* the most predominant; because it contains in its cell wall lipopolysaccharide (LPS) which is common for the whole family (Yoshimura *et al.*, 1999). For the group of Gram positive bacteria, and peptidoglycan (PGN) and lipoteichoic acid (LTA) cell wall representing the repertoire of compounds

recognition similar to (LPS) effects, but with other mediators recognition (Schroder *et al.*, 2003).

*Staphylococcus aureus* is one of the bacteria colonizing the mammary gland tissue destruction effects with high quality and low inflammatory response milk. Its reservoirs scarified skin of the nipple and the stratified epithelium of the nasal mucosa (Kluymants *et al.*, 1997). Current vaccines for *Staphylococcus aureus* are made with whole bacteria and against the PGN (Baselga *et al.*, 1994; Garcia *et al.*, 1996; Kerro-Dego *et al.*, 2006) and the immune response is limited given the numerous factors virulence that this bacterium exhibits (Anderson *et al.*, 2012). However, facilitates recognition of PAMPs low immune response to infection, also facilitates persistent virus infections immunosuppressants as bovine viral diarrhea BVD, IBR Rhinotraqueitis Infectious Bovine viral Bovine Leukemia BVL (Wilson *et al.*, 1997; Barkema *et al.*, 1998).

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The persistence of high risk bacteria in glandular acids such as *Staphylococcus aureus*, *Streptococcus agalactiae*, *Mycoplasma bovis* and some strains of *Escherichia coli* adherent and invasive, are associated with subclinical mastitis cases high (Dopfer *et al.*, 2002). In turn, prepartum mediated immunosuppression stress and chain hormones are in part the result of dysfunction of phagocytosis of polymorphonuclear neutrophil leukocytes which leads to facilitation of colonization of bacteria in glandular structures generates loss and poor quality of milk production (Burvenich *et al.*, 2003; Fox *et al.*, 2005).

The purpose of this study was to analyze the immune response generated application by *intra* - ductal three bacterial antigens in dairy roof racks to determine the presence of specific immunoglobulins against antigens of the formula.

## MATERIALS AND METHODS

### Bacterial antigens

The application of *E coli* obtained from cases of acute mastitis was carried out, as well as four serotypes previously characterized as producers of Pili K-99, 39 and 35 ATCC collection of low and high production of labile thermosensitive and thermosensitive toxins. For Gram + bacteria such as *Staphylococcus aureus* and *Streptococcus agalactiae*, they were obtained from clinical cases of acute mastitis and their respective morphological and biochemical characterization until their characterization. The previously obtained recombinant adhesion protein to the fibronectin (FnB) of *Lactococcus lactis* previously obtained on the wall of *Lactococcus lactis*, was also incorporated as a virulence protein that participates for adhesion to cells and host tissue.

### Selection criteria for pre-calving and intramammary cows

In the region of Atlixco, Puebla-Mexico nine next to dry gestation diagnosed by palpation of about 7 months and low milk production in lactating cows were selected earlier. The herd of 200 breeding receives annual immunization against the virus of the respiratory and reproductive cattle complex, however, no applied biological control of mastitis, this depends on control of hygiene and milking management on a daily basis.

### Analysis of total protein and Immunoglobulins

Of each cow's blood samples were given *Vacutainer* tubes in obtaining serum before and after 30 days of immunization. The serum is centrifuged for 10 minutes at 1,000 rpm, was later deposited in Ependorff tubes and frozen at minus 20 °C. Each sample of 2 cc was evaluated relative to the total protein concentration using a refractometer Brix ATAGO Master-M. For the determination of total protein used 0.5 mL of serum, diluted 1:2 in buffer solution of phosphates (PBS), serum was transferred to a beaker on ice with magnetic stirring continuous, adding 1.5 mL of ammonium sulfate at pH 7.8 up to saturation, subsequently conducted the analysis of the total protein concentration by the Bradford method which is based on the binding of blue dye Coomassie (G-250) to proteins. The acid solution dye is bound to form a protein-dye complex with a higher extinction coefficient than the free dye. This method is sensitive 1-15 mg.

For precipitation total immunoglobulin was used 0.5 mL of serum from each sample of nine dairy cows, ammonium sulfate was incorporated in slowly and under constant stirring for 30 minutes at 4°C for precipitation of globulins. The precipitate was re-suspended by gentle agitation for five minutes and centrifuged at 10,000 G for 15 minutes at 4 °C. The supernatant was discarded and the button is resuspended in 0.5 mL of PBS with a final saturation of 40% salt. Subsequently the fraction of globulins  $\gamma$  was transferred to a dialysis bag against saline 0.85% making 6 changes using barium chloride in a 1:1 with water for 24 to 36 hours at 4 °C until the ammonium sulfate it was no longer detectable. Globulins were preserved frozen at 20 °C.

### Quantification of immunoglobulins in animals immunized by the ELISA method

The ELISA its acronym English *Linked Immuno Sorbent-Enzyme Assay*: is a specific technique to detect Bound antibodies an enzyme capable of generating a product (Lequin, 2005). Indirect ELISA test was developed for the quantitative determination of antibodies in serum in each of dairy cows for the different antigens. 100  $\mu$ L of a  $10^8$  suspension of each bacterium to be tested was added in triplicate on 96-well ELISA plates (Costar). It was left at 4 °C for 12 hours. subsequently they were washed with PBS and applied 100  $\mu$ L of purified immunoglobulins as the

first antibody, incubated at room temperature with moderate stirring for one hour. Do that time passes washed three times with PBS solution and applied 100 µL bovine IgG peroxidase - labeled goat as second antibody. It was read in spectrophotometer for ELISA, Diagnostic Pasteur model LP 400.

**RESULTS AND DISCUSSION**

Immune System immune ma participates significantly in the resolution of infections of viral or bacterial origin. Some germs are producers of compounds that inhibit the immune system, so the chances of the germ (s) in colonizing and invading the host (Nakawa et al., 1993). In the particular case of bacterial infection in the mammary gland in the immediate postpartum period,

polymorphonuclear neutrophils are inhibited in its function of phagocytosis, probably mediated by the stress of labor (Lee et al., 2003). Udders of dairy cows receiving immunization in the drying period, did not have any physical alteration, besides the electrical conductivity of the milk on the fifth day after *postpartum*, it turned with measurements above the cutpoint (300 ± 50), which means that the milk had no alterations of bacterial origin.

*E. coli* is one of the common pathogens found in milk immediate postpartum especially in cows second and third lactation where injury to the sphincter through the nipple are more frequent and loss of function predisposes to colonization of environmental bacteria (Russol et al., 2007). In this research c inco nine cows stimulated *E antigens. coli* showed higher antibody concentration

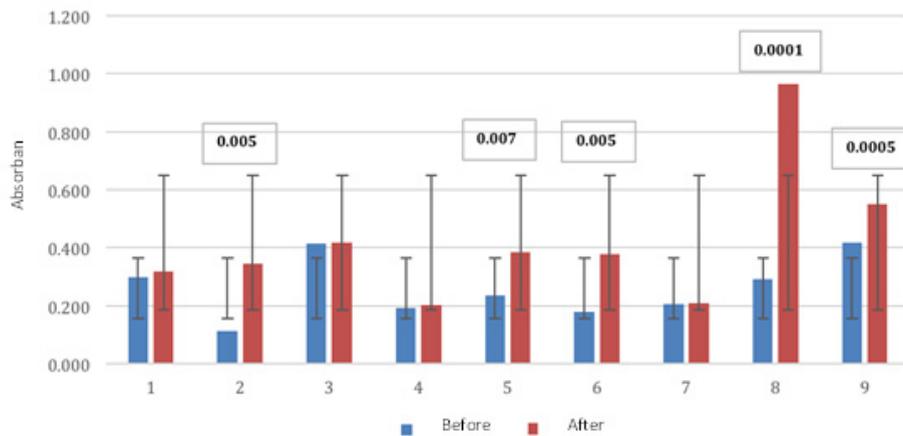


Fig. 1. ELISA absorbance values to *E. coli*.

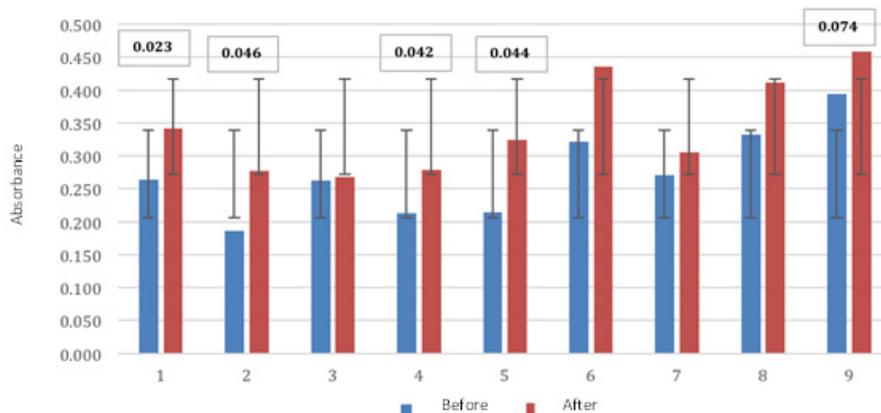


Fig. 2. ELISA absorbance values to *Streptococcus agalactiae*

in statistically significant (Fig. 1). The use of antibiotic products as a curative and/or preventive treatment in the drying stage is a common practice among milk producers and there is little control over their use (Russol *et al.*, 2007). Also the control of antimicrobial resistance of bacteria that infect the mammary gland of cattle is nil or limited in Mexico.

The production of specific immunoglobulins against epitopes of pili and fimbriae of *E. coli* mediated immunization are factors internal support for the facilitation of opsonization, control and resolution to challenges of *E. coli* in cattle milk production (EcoStaph PM + 3 2015) [González *et al.*, 1989]. (EcoStaph PM + 3 2015) [González *et al.*, 1989]. Although *E. coli* serotypes among animals livestock production species maintain their niches regarding

colonization, it is necessary to mention that artisanal producers of fresh bovine milk cheese curdling made without pasteurization what it defines as potential zoonosis to those infections.

*Streptococcus agalactiae* is a bacterium streptococcus group B and other family that prevails in the intestinal tract of various mammals, including humans. In bovines it induces mastitis considered within the pathogens of importance by the reduction of milk production from which its surname derives. It is a germ with high sensitivity to antimicrobial beta lactams, so its treatment both lactating and the drying period eliminates infection (Rossitto *et al.*, 2002), however, the antimicrobial resistance what little is known in Mexico, is a factor that drives the improvement of hygiene and the application of vaccines.

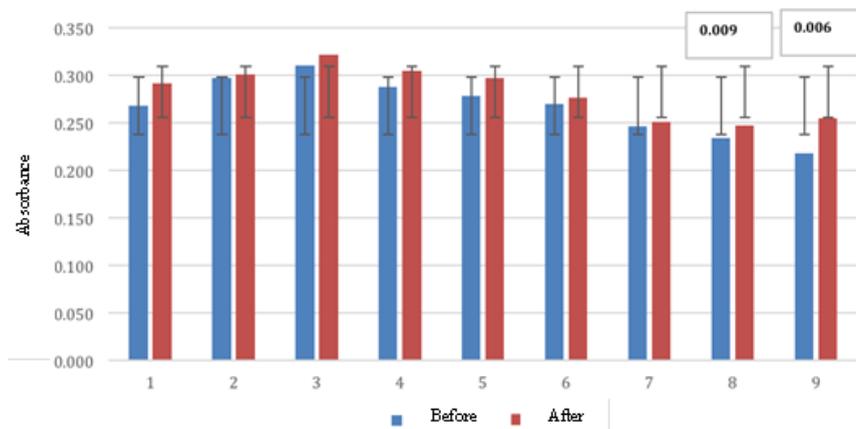


Fig. 3. ELISA absorbance values *Staphylococcus aureus*

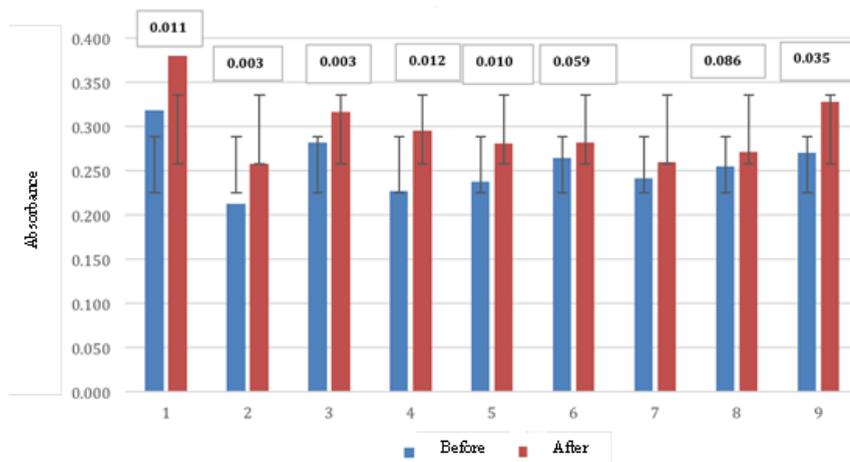


Fig. 4. ELISA absorbance values at (Fnb), *S. aureus* protein Recombinant fibronectin binding

**Table 1.** Protein measurement by refractometer

Sample number	Brix Pre-delivery whey	Brix Postpartum Sera
10	8	10
19	9	10
23	10	11
65	7	9
307	9	9
410	10	10
484	7	12
798	11	11
990	10	10

Yokomizo and Norcross (1978), described the benefits in immunizing vaccine *Streptococcus* by way intra- parenteral and intro-mamaria, other experimental groups developed significant increase in IgG, 1 and 2, IgM and IgA. Furthermore, Lindahl *et al.* (2005) has developed vaccines for *Streptococcus agalactiae* neonatal infections linked to the men, where bovine strains involved are linked to virulence and capsule formation.

In this paper we find that the antigenic stimulus for *S. agalactiae* by way intra tanker, was favorable in eight of nine cows, which responded with good titers of antibodies to this antigen with gnificancia of  $p < 0.0002$  (**Fig. 2**) which confirms previous work by (Yokomizo and Norcross, 1978). Mastitis for *S. agalactiae* is common in ruminants such as; Cows, sheep, goats and monogastricos as; Bristles, bitches, cats, rabbits, etc. significantly reducing the quality of milk (Bergonier, 2014).

The absorbance values in the ELISA test for *Staphylococcus aureus* were concentration values for immunoglobulinas with some statistical significance for 2 of 9 cows (**Fig. 3**). These results are in agreement with previous studies where the low response.

However, stimulation with the virulence protein adhesion to fibronectin *S. aureus*, revealed response in all cows with signi cance rates between  $p < 0.0002$  (**Fig. 4**).

On the other hand, the immuno stimulation via the milk duct, resulted in production of immuno globulins specific G with significance values in; *E coli*, *Streptococcus agalactiae* and Protein FNB of the *S. aureus*.

Antigens applied and its response indicates that the tank via *intra* can be used for the induction of immune response in the drying step. Uncapsulated lymphoid tissue located in the Fürstenberg rosette and permeability of the epithelium of that region suggest sites of antigen presentation to immune cells. It remains to be studied the production of IgA by this route where the mucosal response does with synthesis of this immunoglobulin. *Staphylococcus aureus* bacteria from the group of Gram positive found more frequently in cases of bovine mastitis (Barkema *et al.*, 1998). The infections whose origin was *S. aureus* infections occur with sub clinical or chronic medical that usually persist for the life of the infected animal.

The results described in table 1, show that the sera prepartum of 5 cows were lower compared to sera obtained after delivery. The study by refractometer is often used to assess concentrations of immunoglobulins in the colostrum milk, however, their sensitivity limits are not as accurate as others.

## CONCLUSIONS

The age of the cow, nutritional status, its environment and vaccination schedules will determine the quality and quantity of serum immunoglobulins present in the bloodstream. During the pre-natal period, the cow disseminate their blood capillaries, into the light immunoglobulins of glandular acinos of the mammary gland, forming the protein rich secretion, hormonal factors, lipids, known as colostrum. In cattle, there are particular conditions of immune suppression “stress” pre and post delivery, which favors the invasion of opportunistic pathogens to different organs such as the uterus, vagina and mammary gland (Lloyd, 1993).

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