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Detection of *Staphylococcus aureus* from Clinical and Sub-clinical Bovine Mastitis milk samples using Chicken egg yolk antibodies (IgY)

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Abstract

The egg yolk IgY antibodies were generated against Staphylococcus aureus strains isolated from clinical bovine mastitis milk samples by immunization in 21 week old white leg horn chickens. The protein content of purified IgY showed a gradual increase in protein concentration from twenty first day of immunization, ranging from 0.1 to 3.7 mg/mL of egg yolk and a single protein band with molecular weight of 180 K.Da was obtained by polyacrylamide gel electrophoresis. The titre of antibody was found to be 1: 10000 on 150th day of immunization estimated by ELISA. The S.aureus IgY was specific to S. aureus antigens and showed no cross reactivity with the whole cell antigens of Streptococcus epidermidis, Escherichia coli and Pseudomonas aeruginosa. The egg yolk generated antibodies were tested for its agglutination with four clinical and fifty six normal milk samples, where three clinical and three normal milk samples proved positive agglutination. Simultaneously culturing method of S.aureus was also performed all through the test for the conformation in the analysis.

Key words: Agglutination, Bovine mastitis, S. aureus, Chicken IgY antibodies.

I. INTRODUCTION

Bovine mastitis is an intra-mammary infection that affects large number of dairy cattle throughout the world. It is a most prevalent disease and tends to be a costly disease for dairy producers (Ruegg 2003). Many infectious agents, primarily bacteria, occasionally fungi and protozoa cause mastitis, where the most common contagious mastitis bacterial pathogen is *Staphylococcus aureus.* In clinical cases of mastitis, the herds require an immediate attention or it leads to mortality and the subclinical cases may turn to clinical mastitis (Sears and McCarthy 2003). For appropriate treatment it is vital to identify the contagious bovine mastitis *Staphylococcus aureus* from milk samples of infected cows and normal healthy cows under suspicious of subclinical mastitis.

Identification by culture methods takes minimum of two days. Alternative to culture method is immunological technique, which is rapid method and can be detected within few minutes. The use of rabbit antibodies for diagnosis is costly and is not available commonly for routine diagnosis. The domestic avian species are known to produce higher level of antibodies than mammals without any distress to the animal (Akita and Nakai, 1992) and minimize the number of animals needed. The yolk of the egg receives the same antibodies (IgY) from the maternal chicken serum that can be a suitable alternative source which can avoid bleeding from the animal. The IgG concentration in the blood is 5-6 mg/ml, while in egg yolk is 10 – 25 mg/ml (Leslie & Martin 1973).The concentration of IgY in egg yolk is 1.3 - 1.9 times higher than that in hen blood (Rose et al. 1974, Sunwoo et al. 1996) and further the eggs provide a continuous source of mono specific polyclonal antibody and can be stored at 4^{o} C up to one year.

II. MATERIALS AND METHODS

Source of bacterial strains

The *Staphylococcus aureus* strains were isolated from the milk samples from clinical bovine mastitis of Coimbatore district. The strains isolated were identified by gram reaction, cultural characteristics and biochemical tests.

Preparation of whole cell antigen

The overnight broth culture of *Staphylococcus aureus* was inoculated into Trypticase soy broth and incubated overnight at 37^{o}C on a rotator platform at 150 rpm. Cells were harvested by centrifugation at 6000 rpm for 15 to 20 minutes. Inactivation of antigen was done by addition of half the volume of 1% formalin and incubated overnight at room temperature. Purity of the antigen was checked by sub culturing on to blood agar plates for the nil growth. Prior to immunization the bacterial cells were collected by centrifugation at 10000 g for 15 minutes, washed thrice with saline and resuspended in sterile saline. The antigenic preparations were diluted using saline adjusting the opacity equal to McFarland Barium sulphate standard tube no:1 so as to obtain the final concentration 3 x 10^{8} bacterial cells in the saline.

Immunization and generation of anti-*S.aureus* antibodies in chicken

Twenty one week white egg laying leghorn chickens maintained in the animal house were intramuscularly injected at different sites of breast muscles with 0.5 ml of antigenic preparation. Every seven days after initial immunization the chickens received booster shots with same dose of cell suspension. Test bleeding was done frequently to check for the presence of antibodies in serum. Simultaneously the eggs were collected regularly and stored at 4^{o} C for the isolation and purification of antibodies.

Purification of IgY antibodies from the egg yolk

The egg yolk antibodies were purified by polyethylene glycol and ammonium sulphate precipitation method (Polson *et al*., 1980), the crude IgY was further purified by DEAE cellulose ion exchange column chromatography.

Protein determination

The protein content of the purified IgY fraction was determined by the method of Lowry *et al* (1951). The IgY fraction was then concentrated with poly vinyl pyrolidone (PVP) at room temperature. Purity of the chicken egg yolk antibodies were checked by SDS-PAGE (Laemmli, 1970).

Determination of antibody titer by indirect ELISA

The antibody titer was determined by enzyme linked immunosorbant assay (ELISA) (Hamada, 1989). The antigen solution was diluted with 0.05 M carbonate buffer (pH 9.6) and 100 µl was coated in the microtiter plate wells. The plates were incubated at 4^{o}C for overnight and washed followed by the addition of 200 µl of 1% bovine serum albumin in PBS (pH 7.4) in each well to block the uncoated surface and the wells were washed thrice with 200 µl of PBS-Tween. The IgY from immunized hens and crude IgY from nonimmunized hens (control) were added to the wells and incubated at 37^{o} for 2 hours. After reaction of antigen and antibody, the wells were washed with 200 µl of PBS-Tween. About 100 µl of horse radish peroxidase-conjugated rabbit anti-chicken IgG (Sigma Chemical Co) diluted (1:1000) with PBS-Tween was added to all the wells and the micro titer plate was incubated at 37^{o}C for 2 hours. The wells were washed again with 200 µl of PBS-Tween followed by the addition of 100 µl of TMB solution with hydrogen peroxide. After 20

minutes the reaction was stopped by adding 50 µl of 4N sulfuric acid and the color intensity developed was measured at 490 nm using ELISA reader.

Specificity testing of prepared chicken egg yolk IgY

The specificity of the IgY prepared from chicken egg yolk against *S. aureus* strains isolated from bovine mastitis were checked by slide agglutination test with experimentally prepared whole cell antigens such as *S. aureus*, *Streptococcus epidermidis*, *E.coli* and *Pseudomonas aeruginosa*

Diagnosis of *S.aureus* from milk samples using anti *S. aureus* IgY generated from chickens by slide agglutination test

In the present study, two small holder dairy farms in Annur area were used. About 60 milk samples were collected for the analysis. Out of 60 samples, 4 samples were taken from clinical mastitis based on the physical and clinical appearance of the udder and the remaining 56 samples were taken from normal udder. As a rapid diagnostic tool, the chicken generated *S. aureus* IgY antibodies were used in the present study as an on field test to predict the *S. aureus* mastitis incidence.

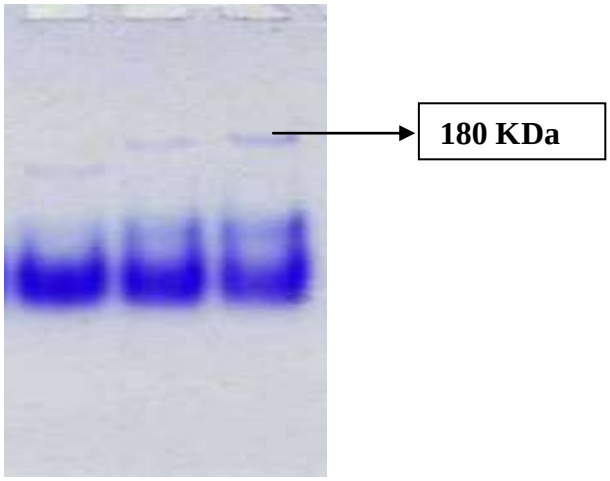
A drop of milk sample from selected cows was placed on a clean glass slide followed by a drop of anti-*S.aureus* IgY. The slide was gently rocked back and forth for one minute and observed for agglutination. The test was repeated thrice to confirm the results. Further, the milk samples with positive agglutination were tested for the presence of *S. aureus* by routine culture method.

III. RESULTS

IgY extraction and purification

The mastitis *Staphylococcus aureus* IgY antibodies were obtained and purified by polyethylene glycol - ammonium sulphate precipitation method and by DEAE cellulose ion exchange column chromatography. Polyacrylamide gel electrophoresis study reveals the presence of single protein band with high molecular weight of 180 KDa (**Fig. 1**) which shows the purity of egg yolk derived IgY generated.

Fig.1. SDS PAGE revealing 180 KDa Protein band (IgY antibody)



Protein estimation

The protein content of the eluted fractions shows a gradual increase in concentration of protein (antibodies) from 21st day of immunization which ranges from 0.1 to 3.7 mg/ml of yolk.

Estimation of antibody titer by ELISA

Agglutination of *S.aureus* antigen and IgY antibody was observed by slide agglutination test. The antibody titer was estimated by indirect antigen capture assay (IACA) and revealed optimum titer of 1:10000 dilution during 150th day after immunization.(**Fig. 2**)

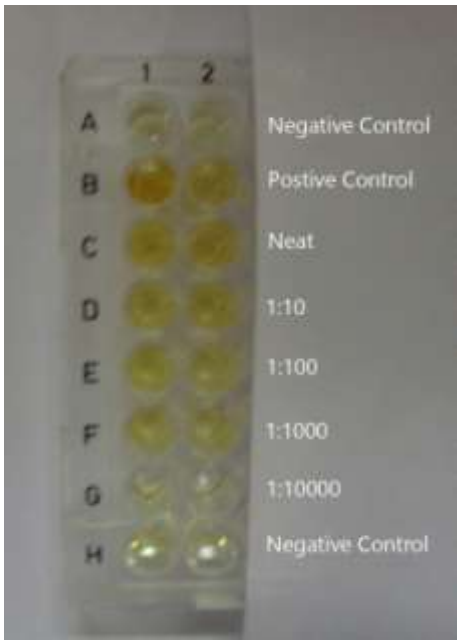


Fig. 2. Indirect antigen capture assay – High titer of 1:10000 was observed during 150th day after immunization

Specificity of egg yolk antibodies (IgY)

Specificity testing of egg yolk IgY showed agglutination only with *S. aureus* whole cell antigen and no agglutination was observed with whole cell antigens of *Streptococcus epidermidis, E.coli* and *Pseudomonas aeruginosa* (**Table.1**)

Table.1. Slide agglutination test

Experimentally prepared whole cell antigens	Agglutination with anti *S. aureus* IgY antibody
Staphylococcus aureus	+
Streptococcus epidermidis	-
Escherichia coli	-
Pseudomonas aeruginosa.	-

(+) Agglutination; (-) No agglutination

Diagnosis of bovine mastitis

Out of 4 clinical mastitis samples the IgY antibodies agglutinate 3 (75 %) milk samples confirming the presence of gram positive contagious *S. aureus* pathogen and out of 56 normal milk samples, 3 samples showed positive agglutination on addition of *S. aureus* IgY on the slide showing the 5.36% of subclinical mastitis. (**Fig. 3**). Out of 60 samples, the prevalence of bovine mastitis was

10% and the incidence of mastitis was also found to be 10%. **(Table.2)**. *In vitro* routine laboratory conformation of agglutinated milk samples were plate cultured for *S.aureus*, which showed positive growth on the plates.

(+) - Agglutination (-) - No agglutination

Fig. 3. Agglutination of *S. aureus* bovine mastitis milk sample with IgY

Table 2. Prevalence of S. aureus mastitis

S. no	Total number of milk samples	Udder condition	No of samples	Number of Positive slide Agglutination with anti- S	Incidence of Clinical S.*aureus* mastitis (%)	Incidence of Sub Clinical S.*aureus* mastitis (%)	Total mastitis incidence (%)	Prevalence (%)
1	60	Clinical mastitis	4	3	75	-	10	10
		Normal udder	56	3	-	5.36		

IV. DISCUSSION

Bovine mastitis is a major concern to dairy producers and the food industry for reasons of farm profitability, food quality, animal and public health. Several methods have been developed to understand the mechanism of pathogenesis, prevention and treatment of bovine mastitis. It is important to identify the infection early for the successful treatment. Rapid identification kits for *S. aureus* have only rarely been specifically evaluated for mastitis diagnostic (Hogan *et al*., 1986).

Among the dairy farmers, milk production is the basic income which gets stumbled when the dairy cattles were found to get mastitis. The present study deals with the prediction of *S.aureus* mastitis as an on field diagnostic test using *S. aureus* IgY antibodies. Among the prevailed mastitis samples collected, our test proved highly positive for both clinical and subclinical mastitis analysis. Broad spectrum antibiotics are always used in the clinical mastitis treatment which paves a way to an emergence of multidrug resistance. *S. aureus* as a contagious mastitis pathogen, an immediate attention is always required both in clinical and subclinical cases. The subclinical mastitis is a dangerous stage of mastitis which cannot be predicted before clinical symptoms. The prediction of

subclinical mastitis using IgY antibodies will prompt the treatment of diseased animal with appropriate treatment by the veterinarians.

S. aureus egg yolk IgY antibodies will enhance the rapid diagnosis of clinical as well as subclinical mastitis. Most of the diagnostic tests are laborious and the *in vitro* culturing takes 2 days for confirmation as well as 1 day for drug choice. Immediate attention and identification of *S. aureus* in the present on field will definitely assure for the remedy or eradication of clinical and subclinical *S. aureus* mastitis. The present study will also help the veterinarians to put an attention on drug of choice and save the dairy sources.

As a diagnostic tool IgY anitibodies are proved to be a success in invitro level for several diseases. In the case of dairy cows the administration of IgY antibodies will not be a successful treatment in the *S. aureus* mastitis. The pathogen forms an exopolysaccaride around its residing area in the milk ducts. The poysaccaride pulp will not be penetrated by any antibiotics or IgY which is a major characteristic feature of this contagious pathogen. Future research works are mandatory to generate antibodies against the *S. aureus* surrounded exopolysaccaride layers in the infected cows.

IV. CONCLUSION

From the study it was concluded that the chicken egg yolk (IgY) antibodies raised against *S.aureus* antigens act as a rapid diagnostic tool in *S. aureus* bovine mastitis at clinical and sub clinical level and would serve as a rapid diagnostic tool in the on field test.

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