

Purification and Characterization of Chicken Egg Yolk Antibodies (IgY) Against Mastitis Causing *Klebsiella pneumoniae*

¹M.S. Uma, ¹M.D. Dinesh, ¹V.M. Anjali, ¹R. Rechana, ¹S. Meenatchisundaram and ²V. Shanmugam

¹Department of Microbiology, Nehru Arts and Science College, Coimbatore, India

²Department of Biotechnology, Nehru Arts and Science College, Coimbatore, India

Abstract: White leghorn hens were immunized intramuscularly with formalin killed *Klebsiella pneumoniae* cells to generate *Klebsiella pneumoniae* antibodies (IgY) in egg yolk. Booster injections of increasing concentrations of antigen were given to raise the antibody level in egg yolk. Amount of protein varied from 0.87 - 7.4mg/ml in egg yolk throughout the immunization period. The antibodies were partially purified from immunized chicken egg yolk by poly ethylene glycol (PEG) and ammonium sulphate precipitation method. Further purification was done by eluting the adsorbed antibodies from DEAE cellulose by linear gradient technique. High titer of more than 1:10000 antibodies were detected by indirect antigen capture ELISA. Inhibition ELISA and growth inhibition assay were also performed to check the effectiveness of IgY. The results indicated that antibodies generated in chicken effectively neutralized mastitis causing *Klebsiella pneumoniae* and has a potential application in diagnosis and treatment of mastitis causing *Klebsiella pneumoniae*.

Key words: *Klebsiella pneumoniae* • Chicken Antibodies (IgY) • ELISA

INTRODUCTION

Mastitis occurs throughout the world wherever dairy cows are found. The continuing presence of the disease may be attributed to deficient management, improper milking procedures, faulty milking equipment, inadequate housing and breeding for ever-increasing milk yield [1]. Unlike contagious forms of mastitis which spread from cow-to-cow during milking, coliforms come from environmental sources, such as manure and organic material/bedding (recycled manure, wood shavings, etc). Coliform bacteria can enter the teat canal both during and between milking. Dirty udders, especially when wet, have enormous bacterial populations. Mastitis, the most important deadly disease of dairy animals is responsible for heavy economic losses due to reduced milk yield (up to 70%), milk discard after treatment (9%), cost of veterinary services (7%) and premature culling (14%) [2]. Milking wet udders and/or teats greatly increases risk of mastitis. In addition, the teat end does not fully close for 1-2 h after milking which can increase the chance of infections immediately following milking.

Mastitis may be classified into subclinical and clinical. Subclinical mastitis is a form of mastitis in which there is no readily detectable changes in the udder itself and no observable abnormality of the milk. Clinical mastitis indicates that there are visible changes in the udder, such as swelling, heat, redness, pain and disturbed functions and or visible changes in the milk, such as clots (gargot) or watery secretions and systemic reactions in varying degrees. Early detection is essential for successful treatment. Peak bacterial numbers have usually already occurred when signs of mastitis appear, so antibiotic therapy is of little to no benefit. In India, the teat dipping as a preventive measure is not regularly practiced by dairy farmers; hence, it is essential to educate the farmers regarding the risk factors of mastitis and also about teat dipping [3].

Drugs necessary to treat infected animals are a direct cause of economic damage, owing to their costs. The cost of drugs varies between countries, depending on the legislation and the infrastructure of the country. Till date broad spectrum antibiotics are injected to reduce financial loss. It leads to serious side effects. Recent research

showed that chicken egg yolk antibodies will act as promising alternative for diagnosis and treatment of mastitis causing organisms. Specific IgY against mastitis-causing *Staphylococcus aureus* inhibits the growth of *S. aureus* and enhances the phagocytosis of *S. aureus* by milk macrophages [4]. Hence our main objective was to evaluate the in vitro activity of egg yolk immunoglobulin (IgY) against mastitis-causing *Klebsiella pneumoniae* which is prevalent during rainy season in Coimbatore district.

MATERIALS AND METHODS

Experimental Animals: Twenty four week old, single comb white leghorn chickens obtained from L.K. Poultry Farm, Coimbatore were maintained in our animal facility. These were used in the study for the production of *Klebsiella pneumoniae* antibodies (IgY).

Isolation and Identification of Mastitis Causing *Klebsiella pneumoniae*: Mastitis milk samples were collected from various veterinary dispensaries in and around Coimbatore during rainy season. After collecting the milk samples, isolation and identification of bacteria was done on the basis of morphological, cultural and biochemical characteristics. The isolates were identified as *Klebsiella pneumoniae*.

Preparation of Antigen: Mastitis causing *Klebsiella pneumoniae* cells were grown overnight in nutrient broth. Cells were separated by centrifugation, washed and suspended in phosphate-buffered saline (PBS) at a density of 10^{10} cells/ml. Formaldehyde was added to a final concentration of 3% (vol/vol) and the suspension was kept around 4°C for 16 h. Cells were washed twice with PBS to remove the formaldehyde and resuspended in sterile PBS pH 7.2 [5]. Complete killing of the *Klebsiella pneumoniae* suspensions was confirmed by culture. Bacterial suspensions were stored around 4°C before use.

Development of Anti- *Klebsiella pneumoniae* Antibodies in Chickens: Formalin killed *Klebsiella pneumoniae* cells were dispersed separately in sterile 0.9% phosphate buffered saline (PBS) in the concentration of 10^3 cells/ml. These antigens were injected intramuscularly at the multiple sites of breast muscles of 24-week-old white leghorn chickens. Chickens received subsequent booster

injections with increasing concentration of antigens (10^3 - 10^8 cells/ml) at 15 days interval by the same route of administration [5]. Blood was sampled at intervals of two weeks of immunization and checked for the presence of antibodies. Eggs were collected from day 0 until the end of the experiment and stored at 4°C.

Purification of Antibodies from Egg Yolk: The antibodies were extracted from egg yolk by the method of Polson *et al.* [6] using polyethylene glycol and ammonium sulphate precipitation method. Briefly, the egg yolk was separated from white, washed with distilled water to remove as much albumin as possible and rolled on a paper towel to remove adhering egg white. The membrane was punctured and the yolk without the membrane was allowed to flow into a graduated cylinder. An equal volume of buffer S (10 mM phosphate, 100mM NaCl, pH 7.5, containing 0.01% sodium azide) was added to the yolk and stirred. To this mixture, 10.5% PEG 8000 in buffer S was added to a final concentration of 3.5%. The mixture was stirred for 30 minutes at room temperature and centrifuged at 11000 rpm for 20 minutes. The supernatant was filtered through double-layered cheesecloth. The 42% PEG in buffer S was added to make a final concentration of 12% PEG. The mixture was stirred thoroughly and centrifuged at 11000 rpm for 20 minutes. The pellet was dissolved in buffer S to the original yolk volume and an equal volume of 4M ammonium sulphate (pH 7) was added and the resultant was centrifuged at 11,000 rpm for 20 minutes, the pellet was dissolved in 1ml of buffer S (without NaCl) over night. The content was desalted by the dialysis process.

DEAE Ion Exchange Column Chromatography: The crude fraction of IgY obtained was further purified by DEAE cellulose ion exchange column (1.0 x 60 cm, Sigma, USA) chromatography. The column was packed with DEAE cellulose and equilibrated with 25mM phosphate buffer pH 8.0. IgY antibodies were loaded and washed thoroughly with 25mM phosphate buffer pH 8.0. The column was eluted by passing linearly increasing sodium chloride (0- 2M) through the column. Three milliliter fractions were collected at a flow rate of 0.6ml/min. Total protein was estimated by Lowry's method [7]. The IgY fraction was then concentrated with poly vinyl pyrrolidone (PVP) at room temperature. The purity of chicken egg yolk antibodies was checked by SDS-PAGE [8].

Determination of Antibody Titer by Indirect Elisa: The titer of the antibodies generated against *Klebsiella pneumoniae* was determined by indirect antigen capture ELISA [9]. Nunc polysorp ELISA plates were coated with formalin killed *Klebsiella pneumoniae* antigen using coating buffer (0.05M carbonate bicarbonate buffer, pH 9.6) and incubated at 4°C overnight. The above plates were washed with PBST for 3 times. The empty sites were blocked by 1% BSA (200 µl / well) and incubated at 37°C for 1 hour. Plates were subsequently washed and incubated with anti- *Klebsiella pneumoniae* (100 µl / well). PBST and pre immune sera served as controls. Wells were washed thrice with PBST and 100 µl of diluted (1:1000) rabbit anti chicken immunoglobulin coupled to Horse Radish Peroxidase (Genei Pvt Ltd, Bangalore) was added and incubated. These plates were washed and 100 µl of TMB/H₂O₂ (Genei Pvt Ltd, Bangalore) was added. The plates were allowed to stand at room temperature in dark for 20 minutes. The reaction was stopped by adding 50 µl of 4N sulphuric acid and plates were read at 490 nm in an ELISA reader. All assays were done in triplicates.

Inhibition Elisa: Various concentrations of chicken egg yolk antibodies (IgY) raised against Mastitis causing *Klebsiella pneumoniae* were pre-incubated with overnight cultures of *Klebsiella pneumoniae* and added to microtiter plate wells previously coated with *Klebsiella pneumoniae* antigen. After washing steps the bound antibodies were reacted with anti rabbit chicken immunoglobulin coupled to Horse Radish Peroxidase and assayed with OPD and H₂O₂ [5]. The reaction was stopped by adding 50 µl of 4N sulphuric acid and plates were read at 490 nm in an ELISA reader.

Micro Agglutination Test (MAT) and Microscopic Slide Agglutination Test (MSAT): For MAT test the bacterial cell suspension was adjusted to a desired optical density 0.42 at 620 nm. In a microtiter plate two rows were assigned for the test, 80µl of sterile saline was added in the first well of each row and 50µl was added in the remaining wells of each row. Then 20µl of *Klebsiella pneumoniae* IgY was added in the first well of the first row and doubling dilution was done by transferring 50µl from the first well to second well, second to third and so on. The same procedure was repeated in the second row (control) but except *Klebsiella pneumoniae* IgY, 20µl of non specific antibody solution was used. Finally 50µl of bacterial cell suspension was added to all the wells of

each row and incubation was done at 25°C for overnight [10]. After incubation, the plate was observed and the titer was defined as the highest dilution of *Klebsiella pneumoniae* antibody showing agglutination by comparing with controls. Microscopic slide agglutination test was also performed.

Growth Inhibition Assay: Growth inhibition assay was carried out by the method described by Marco Cesar *et al.*, 2009. [11]. This experiment is to check whether the anti- *Klebsiella pneumoniae* IgY could inhibit *Klebsiella pneumoniae* growth in liquid medium or not. *Klebsiella pneumoniae* was inoculated into 5ml Todd hewitt Broth (THB) and into another 5 sets of 5 ml of THB containing increasing concentrations of chicken egg yolk antibodies (1 - 5µg/ml of IgY) and incubated overnight at 37°C. PBS alone served as negative control and 100 µg/ml of ampicillin was the positive control. After incubation the contents were sub cultured onto nutrient agar and incubated overnight at 37°C. The tubes were also checked for OD and turbidity method to check the growth inhibition of *Klebsiella pneumoniae* by chicken egg yolk antibodies (IgY).

RESULTS

Mastitis causing *Klebsiella pneumoniae* were isolated from mastitic milk. The egg yolk antibodies were purified by polyethylene glycol and ammonium sulphate precipitation methods. This partially purified IgY was further purified by DEAE cellulose column chromatography (Fig. 1). Microscopic slide agglutination showed agglutinating antibodies in both serum as well as egg yolk's of immunized white leghorn chickens. In the micro agglutination test the agglutination was observed up to 1:1280 dilution. Determination of the titer of antibodies in the immunized chicken egg yolk was carried out by indirect antigen capture assay (IACA), indicating that the amount of specific IgY in the egg yolk significantly increased when the chickens received booster doses at regular time intervals. High peak titer of 1:10000 was observed during 63rd day of observation (Fig. 2). A single protein band of high molecular weight (180KDa) was observed in SDS-PAGE showed the purity of IgY (Plate 1). There was a decrease in *Klebsiella pneumoniae* growth with increasing concentration of antibodies (IgY). The growth was completely inhibited when 3µg/ml of IgY was present in the culture.

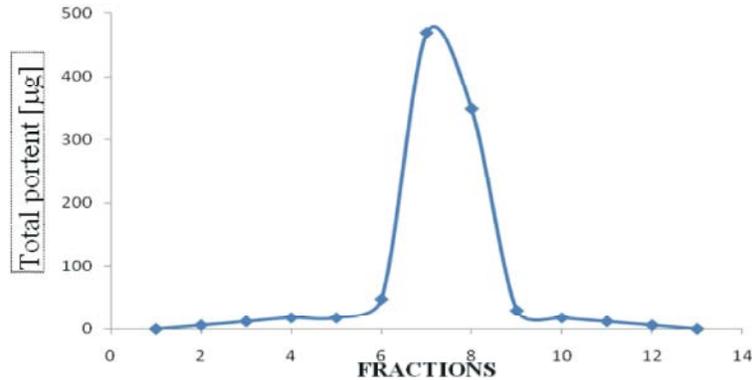


Fig. 1: Column Chromatography Using DEAE Cellulose

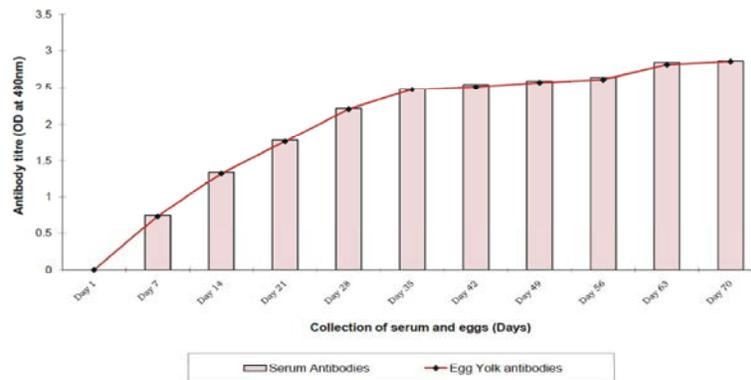


Fig. 2: Kinetics of antibody production in hens immunized with *Klebsiella pneumoniae*.

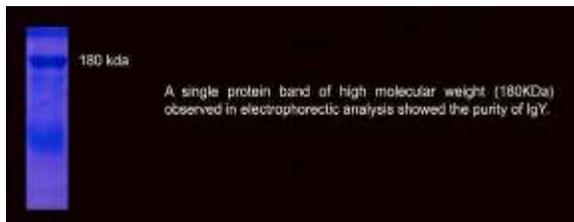


Plate 1: Protein profile of IgY by SDS-PAGE

In inhibition ELISA there was decrease in absorbance with increasing concentration of IgY. It indicated that chicken egg yolk antibodies (IgY) effectively neutralized the antigens during incubation period.

DISCUSSION

Mastitis has been continuously recognized as one of the major disease problems concerning the dairy industry. It is also one of the most costly diseases confronting the dairy farmers. Estimating economic losses resulting from mastitis becomes an extremely difficult task because of the

many levels of infection and other factors. Coliform bacteria are responsible for a great number of acute clinical mastitis cases in dairy cows. Mastitis is a global problem as it adversely affects animal health, quality of milk and considerable reduction in milk production and every country including developed ones suffer huge financial losses [12]. De and Mukharjee [13] reported that the overall prevalence of clinical mastitis and subclinical mastitis are 15.18 and 42.93% respectively during the month of July and August in Uttar Pradesh.

Early detection is essential for successful treatment. Antimicrobial therapy is pivotal for its containment and recovery. Despite the wide spread use of these drugs, antimicrobial treatment of mastitis has been less effective than desirable in short period of time. Recent research showed that chicken egg yolk antibodies will act as promising alternative for diagnosis and treatment of mastitis causing organisms. The present investigation showed that the chicken egg yolk antibodies (IgY) inhibited the growth of mastitis causing *K. pneumoniae*. A high peak of titer of 1:10000 was obtained for the DEAE

column purified egg yolk antibodies by ELISA. In the growth inhibition assay, there was decrease in bacterial growth when the specific egg yolk antibodies were added to the *K. pneumoniae* culture. The growth was completely inhibited when 3µg/ml of IgY was added to the culture. Similarly the growth of *S. aureus* was inhibited by the specific IgY at concentrations of 1-5 µg/ml [11]. Hens therefore produce a more hygienic, cost efficient, convenient and sufficient amount of antibodies compared to the traditional method of obtaining antibodies from mammalian serum [14]. A major advantage of using birds is that the antibodies can be harvested from the egg yolk instead of serum, thus making blood sampling obsolete. The purified antibodies also retained their antigen binding capacity after 6 months at +20°C or 1 month at +37°C. An egg can be stored in +4°C, with just a small loss of IgY activity for at least six months [15].

The present experimental results indicated the antigen specific chicken egg yolk antibodies (IgY) effectively inhibited the mastitis causing *K. pneumoniae*. The antibody generated was potent enough in inhibiting the growth of *K. pneumoniae*. The above result implicates that highly purified chicken egg yolk antibodies could be used for therapy in bovine mastitis. Chicken egg yolk antibodies will play an increasing role in research, diagnostics and immunotherapy in future.

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