South Asian Journal of Research in Microbiology

9(3): 1-15, 2021; Article no.SAJRM.67285
ISSN: 2582-1989

Potential for Neutralizing Chicken Egg Yolk (Igy) Generated against Mastitis Bovine, Caused by *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*

R. Mahenthiran[1*], K. V. Sri Alamelu[2] and Sornambiga Ravi[2]

[1]Department of Microbiology, Dr. N. G. P. Arts and Science College, Coimbatore, Tamilnadu, India.
[2]Post Graduate Research Department of Microbiology, Dr. N. G. P. Arts and Science College, Coimbatore, Tamilnadu, India.

Authors' contributions

This work carried out in collaboration among all authors. Author RM designed the study. Authors KVA and SR performed the field work and laboratory work. All authors managed the literature and manuscript searches. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/SAJRM/2021/v9i330208
Editor(s):
(1) Dr. Ana Claudia Coelho, University of Tras-os-Montes and Alto Douro, Portugal.
Reviewers:
(1) Joilson Silva Lima, Instituto Federal de Educação, Ciência e Tecnologia do Ceará (IFCE), Brazil.
(2) Pharaoh Fellow Mwale, Taipei Medical University, Taiwan.
Complete Peer review History: http://www.sdiarticle4.com/review-history/67285

Original Research Article

Received 22 February 2021
Accepted 28 April 2021
Published 06 May 2021

ABSTRACT

Introduction: Mastitis is a complex disease, defined as an inflammation of parenchymal cells of mammary glands. It is a most common disease in dairy cattle. More than 250 microbes are found to cause mastitis. Recent report shows that total loss of about $2295/month in worldwide due to mastitis.
Researchgap/Challenges: Chicken egg yolk immunoglobulin (IgY) provides an inexpensive and effective source of antibodies for the passive immunization of animals. It is a promising alternative for the treatment and prevention of bacterial infections, and has shown to be effective against a number of pathogens.
Aim: To develop an inexpensive and effective source of antibodies by using chicken egg yolk against mastitis caused by *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* in bovine.
Methodology: Bovine mastitis infected milk sample was collected in and around Coimbatore, India. Initial step was to isolate and identify the microbes like *Klebsiella pneumoniae* and

**Corresponding author: Email: mahenthiran@drngpasc.ac.in;*

Pseudomonas aeruginosa from milk samples of cattle infected with mastitis. Then to prepare and standardize the whole cell antigen of *Klebsiella pneumonia* and *Pseudomonas aeruginosa* followed by generation of antibodies against the prepared antigens in 21-weeks old white Leghorn chickens. Next step includes purification and characterization of anti-*Klebsiella pneumoniae* IgY and anti - *Pseudomonas aeruginosa* IgY antibodies from the egg yolk of immunized chickens. Finally, to evaluate the specificity of the purified IgY by Indirect ELISA and to measure the inhibitory activity of the IgY against the pathogens by growth inhibition assay.

Results: Cell antigen was prepared. Anti- *Klebsiella pneumoniae* IgY and anti - *Pseudomonas aeruginosa* IgY were recovered from chicken egg yolk that was detected by protein estimation (Lowry et al technique) and ELISA. In Lowry et al method (1951) total anti *P. aeruginosa* IgY was found to be 0.511 mg ml^{-1} and total anti *K. pneumoniae* IgY was found to be 0.522 mg ml^{-1}.

Conclusion: Generated antibodies were identified as very specific to the immunization antigen. Based on invitro studies generated antibodies were specific to immunized antigen. It concluded that it will be useful or therapeutic after further conformation.

Keywords: Klebsiella pneumonia; Pseudomonas aeruginosa; IgY; SDS-PAGE; growth inhibition assay; ELISA.

1. INTRODUCTION

Mastitis is a complex disease due to atmospheric changes, defined as an inflammation of parenchyma of mammary glands. It is characterized by physical, thermal, chemical, mechanical and usually bacteriological changes in milk changes in microbial flora in glandular tissues with prevalence of pathogenic organism. It is the most common and most costly disease of dairy cattle [1].

Mastitis causes heavy economic losses to the dairy industry worldwide. The first report on mastitis caused losses in India was about Rs.529 crore annually [2-6]. Recent report of milk total losses to \$2295 $month^{-1}$ in world wide. Nearly 70 % of this loss is a result of reduced milk production caused by sub-clinical [7-11]. Apart from its economic importance, it is also a matter of concern of carries public health significance. Moreover, presence of antibiotic residues in the milk is undesirable due to its public health concern [12-17]. Traditionally, the mastitis control programmes are focused at use of chemical disinfectants, antiseptic or herbal tea dips and antibiotic therapy [18-24]. In herds without an effective mastitis control program, about 40% of the cows are infected in an average of two quarters [25-32]. Reduced milk production accounts for about 70% of the total loss associated with mastitis. The inflammatory response consists of an increase in blood proteins and white blood cells in the mammary tissue and the milk [33].

The antibiotic treatment may help in minimizing the losses, but simultaneously may lead to drug resistance. Factors such as pharmacokinetic problems, and phagocytosis depressing effect of certain antibiotic and appearance of residue in milk, restricts the success of antibiotic therapy [34-41]. When an antibiotic treatment is recommended, it is very critical to follow instruction, especially regarding the duration of treatment [42-44]. Only mastitis caused by *S. agalactiae* can be treated successfully, with antibiotics during lactation. Additional losses are associated with changes in milk quality and composition of milk. The possibilities of drug residues in milk also increases [45].

Since chicken IgY does not cross-react with mammalian IgG and does not bind bacterial or mammalian Fc receptors non-specific binding is reduced, and the need for cross-species immune absorptions also is eliminated [46-56]. Subclinical mastitis is believed to be more prevalent than clinical mastitis in most countries [57-65]. Sharma and Mukharjee (2009) have individually reported the overall prevalence of clinical mastitis and subclinical mastitis as 15.18% and 42.93% respectively during the month of July and August in Uttar Pradesh, and 42.18% and 10.93%, respectively, in dairy cows in Jammu [66].

A study in Jammu by Sudan *et al.* [67] suggests that *Staphylococcus aureus* is major pathogen (56.89%), followed by *Micrococcus* spp. (15.51%), *Bacillus cereus* (12.06%), *Staphylococcus epidermidis* (8.62%), *Klebsiella* spp. (3.44%), *Escherichia coli* (1.72%), and *Pseudomonas* spp. (1.72%). A total of 55 samples (8.2%) were positive for both *Klebsiella* spp and *Pseudomonas* spp. The most prevalent mastitis Enterobacteriaceae were found in 67

samples (10.0%) most of which were positive for coliform enterobacteria (56samples,8.4%). Among coliform enterobacteria, *K.pneumoniae* prevailed in 44samples;(6.6%). P. aeruginosa was isolated from 12(1.8%) samples. (Cucarella *et al.,* 2010). Hence the present investigation focuses on generation of antibodies against *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* separately, which could be used for the treatment of two different diseases at single attempt by mixing together as consortium as the future progression of this study.

Purpose of study: Chicken egg yolk immunoglobulin(IgY) provides an inexpensive and effective source of antibodies for the passive immunization of animals. It is a promising alternative for the treatment and prevention of bacterial infections, and has shown to be effective against a number of pathogens.

2. MATERIALS AND METHODS

2.1 Growth and Maintenance of Standard Strain

Milk samples that were subjected to cause mastitis were collected from cows in and around Coimbatore. Milk samples were collected aseptically and transfer to the laboratory milk for culturing and biochemical tests. Purity of the strain was maintained by plating the overnight grown culture on respective nutrient agar plates. Isolated saline colonies were verified microscopically before future sub culturing or storage.

2.2 Experimental Animal

Twenty-one weeks old e health was obtained from Chandran poultry farm, Palladam. The birds were maintained free from specific pathogen and were fed with layer mesh.

2.3 Characterization of Organism

To check for purity of the culture, microscopic and biochemical characterization of the strains were carried out by the following tests.

2.3.1 Microscopic characterization-Gram staining

The prepared smear was air dried and heat fixed. Crystal violet was flooded over the smear for one minute and drained. After washing Gram's iodine was added and left for one minute. The smear was then washed in 95% (V/V) ethanol for 30 sec. It was counter stained with safranin for two minutes. After air drying, the smear was examined under oil immersion objective in a light microscope.

2.3.2 Cultural characterization of organism-Gram staining

The *K. pneumoniae* and *P. aeruginosa* were sub cultured MacConkey agar, Cetrimide agar and Nutrient agar plates. Plates were incubated at 37ºC overnight and cultural characteristics of the colonies were studied after incubation.

2.3.3 Biochemical characterization

Standard strains were further characterized by some biochemical tests which include – oxidase, catalase, indole, Methyl Red-Voges Proskauer (MRVp), citrate utilization urease test. Bacterial cultures were inoculated in the biochemical media and incubated at 37ºC for 24 h. After incubation the tubes were observed and results were noted.

2.4 Preparation of Whole Cell Antigen

2.4.1 *Klebsiella pneumoniae and Pseudomonas aeruginosa*

Pure isolated colonies of *K. pneumoniae* and *P. aeruginosa* were grown in 5mL of Luria-Bertani broth at 37ºC for overnight. They were then transferred into 250mL of Luria-Bertani broth and incubated overnight at 37ºC and cells were harvested by centrifugation at 5,000 rpm for 15 to 20 min. The harvested cells were resuspended in 15 mL of physiological saline. Half the volume of 3.7% formalin was added and refrigerated at 4ºC for 24 hours with mixing in between then formalin was removed by centrifugation. The pellets were resuspended in saline and stored under refrigeration.

2.4.2 Purity testing of antigen

Complete killing of the bacteria was tested by resuspending an aliquot of the cell pellet in PBS saline and plating 100ul of this suspension into nutrient agar and MacConkey agar medium. The plates were incubated overnight at 37ºC and examined for the presence of bacterial growth.

2.5 Immunization of Chickens

For first immunization, the five-month-old white leghorn chickens were intra muscularly injected

at multiple sites of the breast muscles with prepared bacterial antigens. Booster doses were given with two weeks intervals. Blood was sampled at intervals of two weeks from the initiation of immunization and checked for the presence of antibodies. Further, eggs laid by the chicken under the test were collected regularly and stored at 4ºC

2.6 Purification and Concentration of Egg Yolk Antibodies

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. The egg yolk antibodies were purified by the method of Polson *et al*. [68]. To the 20mL of egg yolk, an equal amount of buffer "S" (10mM phosphate, 100mM NaCl, pH 7.4 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. The stirred mixture was centrifuged at 11,000 rpm for 20 minutes. The supernatant was filtered through double-layered cheese cloth. The 42% PEG in buffer- "S" was added to make final concentration of 12.5% PEG. The mixture was stirred thoroughly for 30 min at room temperature and centrifuged at 11,000 rpm for 20 min. The pellet was re-dissolved in buffer "S" to the original yolk volume and equal volume of 4M Ammonium sulphate (pH 7) was added and incubated at 0°C for 30 min. The solution was centrifuged at 11,000 rpm for 20 min. The precipitate was re-dissolved in buffer- "S" (without NaCl) and was desalted by dialysis to remove ammonium sulphate.

2.6.1 Purification of IgY fraction by dialysis

The egg yolk antibodies were desalted against buffer "S" (without NaCl) to remove ammonium sulphate. The cellulose membrane tubing was cut into pieces of required and convenient length and allowed to boil for 10 min in a large volume of 2% (w/v) sodium bicarbonate and 1mM of EDTA (pH 8.0). The tubing was rinsed thoroughly in distilled water. Again, it was allowed to boil in 1mM EDTA (pH 8.0). The tubing was cooled down and stored at 4°C. Care was taken that the tubing was thoroughly submerged. After this step, the tubing was handled with gloves. Before use, the tubing was washed inside and outside with distilled water. The pooled IgY fraction obtained from egg yolk was transferred to an active dialysis bag. The contents were pooled into the dialysis bag and clipped with dialysis bag clips after including some air and twisting the open end of the dialysis bag. The bags containing pooled contents were dispensed into buffer respective buffer for dialysis process. The dialysis maybe carried out in 1-2 L of phosphate buffer for over 16 hours with 2 times replacement of buffer. 2-4 drops of Nessler's reagent were added to check the completion of dialysis. The dialysis is carried out until the brown colour formation stops.

2.7 DEAE Cellulose Ion Exchange Column Chromatography

The chicken egg yolk antibodies were purified by DEAE cellulose Ion Exchange Column Chromatography. 25-30 g of pre-swollen (with distilled water) DEAE spadix A-50 cellulose was added to a beaker containing equal volume of 0.1N NaOH, stirred well in such a way that no air bubbles were formed and left at room temperature for 30 min. The supernatant was decanted and the sediment was treated with distilled water, stirred well and left at room temperature for 30 min. This was repeated several times until there was a neutral reaction. Further, the sediment was mixed with equal volume of 0.1N HCl and was left at room temperature with intermittent stirring for 30 min. This was further washed several times with distilled water until there was a neutral reaction

2.7.1 Packing of chromatography column

For purification of immunoglobulin, the column size 2x30cm was used. The burette was first cleaned well and it was packed first with glass wool to form an even bed and a rubber tube with pinch-cock was attached to the tip of the burette. The column was fixed to stand in vertical position. The DEAE slurry was poured into the column along the sides to avoid air bubbles and was allowed to settle. Once the column was set, it was equilibrated with 25mM phosphate buffer (till the out flow of buffer showed pH 8.0).

2.7.2 Sample application and elution

Once the column was equilibrated (25mM phosphate buffer, pH 8.0) the level of buffer in column was allowed to run down to the matrix and the outlet was closed. The immunoglobulin IgY sample layered on the top of the column and

was allowed to run till all the sample had entered the bed. Then a continuous constant flow of 25mM phosphate buffer was maintained until all the un retained protein came out. IgY was eluted with 250mM phosphate buffer pH 8.0.

2.7.3 Concentration of antibodies

The eluted IgY fractions were pooled together and concentrated by freeze drying. The freeze dried IgY powder was stored under refrigeration and used for further studies. The concentrated protein was then checked for protein estimation Lowry *et al*., (1951) and the purity of IgY was analysed by SDS-PAGE according to the method of Laemmli (1970) and the IgY content was estimated.

2.8 Protein Profile of IgY

Protein profile of IgY antibodies were analysed by Sodium Dodecyl Sulphate Ploy Acryl Amide Gel Electrophoresis (SDS-PAGE) as described by Laemmli (1970). According to Laemmli (1970) the proteins are resolved with 10% (W/V) polyacrylamide separating gel and 4% (W/V) poly acryl amide stacking gel at 250V and 10mA.Equal ratio of prepared bacterial antigens (30µL) and sample treating buffer (30µL) were mixed well and loaded into sample wells. A wide range molecular weight (6.5-205 KDa) marker was also run along with the proteins. The sample was run until they reach the bottom of the gel. The characteristic protein pattern for the IgY antibodies can be visualized after Coomassie brilliant blue staining.

2.9 Titration of Antibodies by ELISA

The antibody titre was assayed by an ELISA procedure as described by Sun woo *et al.* (2000) with modifications. The specific binding activity of IgY against whole bacterial cells was tested as follows. A microtiter plate was coated with 100 *µ*L of *P. aeruginosa* ($2x10^6$) and *K. pneumoniae* ($1x10^7$ cells per well) whole cells in carbonate-bicarbonate buffer (0.05 *M*, pH 9.6). The specific egg yolk antibodies (IgY) diluted from 1:10 to 1:1,00,000 was reacted with coated antigens. The same volume (100 *µ*L) of rabbit anti-chicken IgG conjugated with horseradish peroxidase (diluted 1:5,000 in PBS) and freshly prepared substrate solution, TMB in 0.05 *M* phosphate citrate buffer (pH 5.0) containing 30% hydrogen peroxide were used for secondary antibody and substrate, respectively. Absorbance of the mixture was read at 490nm by a ELISA micro plate reader.

2.10 Specificity of Antibodies by Growth Inhibition Assay

Growth inhibition assay was conducted to investigate whether the anti- *K. pneumoniae* and *anti- P. aeruginosa* IgY could inhibit *K. pneumoniae* and *P. aeruginosa* growth in liquid medium as described by the method of Zhen with some modifications (Zhen *et al*. 2008). The extracted specific IgY was dissolved in Trypticase soy broth at concentrations of 5,10 and 20 mg/ml. TSB with 20mg/ml of nonspecific IgY and without IgY was the positive control and that with 100 µg/ml of chloramphenicol was the positive control. All preparations were filter sterilized using 0.2−µm filter and incubated with 2.7×10^7 CFU/mL of *K. pneumoniae* and *P. aeruginosa* at 37°C with shaking. The suspensions were transferred to a microtiter plate (100 µL per well) at 2-h intervals until the OD600nm reached OD of stationary phase. The OD value was monitored using a microtiter reader. The growth inhibition assay was conducted for 12 h.

3. RESULTS AND DISCUSSION

3.1 Characterization of *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*

3.1.1 Gram staining

The gram stained smear of the strain *P.aeruginosa* showed the gram-negative rod and 1.5 to 3 x 0.5 µm in diameter with oxidative and this large motile bacterium. *K. pneumoniae* showed the Gram-negative rod and 0.5 to 2.0 µm in diameter with a thick, surrounding capsule. This large, non-motile bacterium.

3.1.2 Cultural characteristics

3.1.2a *Klebsiella pneumonia*

T*he* isolated bacterial cultures were grown on Nutrient agar. The colony morphology was studied on Nutrient agar and MacConkey agar. This produces large sticky colonies when plated on nutrient agar medium. Grey, round, shiny and mucoid colonies on MacConkey agar medium.

Table 1. Immunization schedule

IMMUNIZATION	Antigen Dosage ($1x10^8$ CFU/mL)	
	K. pneumoniae	***P. aeruginosa***
BOOSTER DOSE I	1mL	1mL
BOOSTER DOSE II	1mL	1mL
BOOSTER DOSE III	1mL	1 mL
BOOSTER DOSE IV	1mL	1 mL

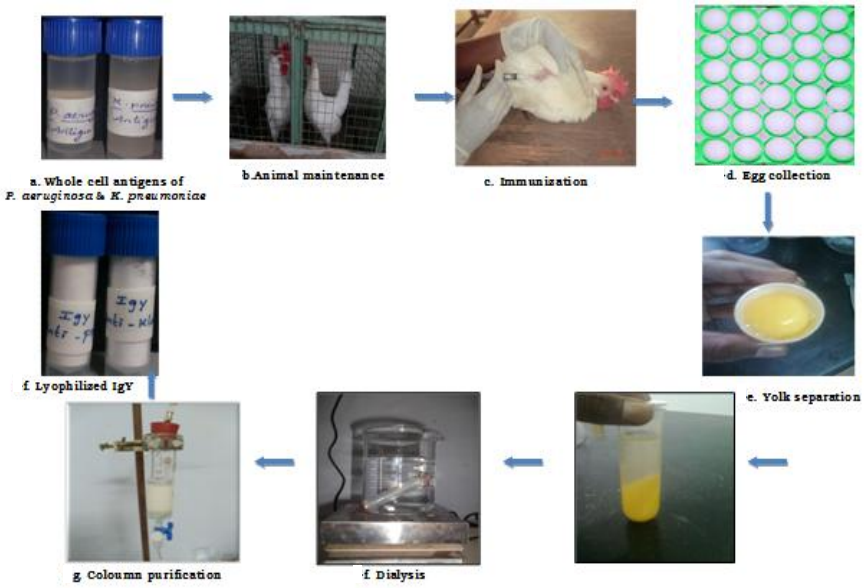

Plate 1. Generation and purification of anti-*P. aeruginosa and anti- K. pneumoniae* IgY process

Table 2. Biochemical characteristics of *K. pneumoniae and P. aeruginosa*

S. No	BIOCHEMICAL TEST	RESULT	
		K.pneumoniae	*P.aeruginosa*
1	Indole test	Negative	Negative
2	Methyl Red test	Negative	Negative
3	Voges-Proskauer test	Positive	Negative
4	Citrate test	Positive	Positive
5	Catalase test	Positive	Positive
6	Oxidase test	Negative	Positive
7	Urease test	Positive	Positive

3.1.2b *Pseudomonas aeruginosa*

The isolated bacterial cultures were grown on Nutrient agar. The colony morphology was studied on Nutrient agar and cetrimide agar media. This produces large colonies with green colour pigment produces when plated on nutrient media. It produces a number of water-soluble pigments. When the yellow-green pyoverdine combines with the blue water-soluble pigment pyocyanin, the bright green colour characteristics on cetrimide agar medium

3.1.3 Biochemical characteristics

Biochemical characteristics of the bacterial strain were determined by using various biochemical tests.

3.2 Generation of Chicken Egg Yolk Antibodies in White Leghorn Hen

The 21-week-old white leghorn Hens were immunized intramuscularly with prepared bacterial antigens to generate anti-

K.pneumoniae and anti-*P.aeruginosa* antibodies with two-week intervals for booster doses. The eggs were collected stored at 4°C and antibodies were separated from egg yolk. The method used for purification of chicken egg yolk antibodies were PEG and ammonium sulphate precipitation described by Polson et al. [68]. The precipitate was desalted by dialysis to remove ammonium sulphate. The egg yolk antibodies were further purified by ion exchange chromatography using Anion exchanger like DEAE – Cellulose and immunoglobulin fractions were recovered. The recovered antibodies were detected by Protein estimation and ELISA.

3.3 Protein Estimation

The total protein and anti-*P. aeruginosa* IgY concentration was estimated by Lowry *et al.,* (1951). The Total protein concentration of antibody was found to be 8.022 mg /mL and total anti *P. aeruginosa* IgY was found to be 0.511 mg/ml at sixth weeks in the day after immunization. The total protein and anti- *K. pneumoniae* IgY concentration was estimated by Lowry *et al.,* (1951). The Total protein concentration of antibody was found to be 7.997 mg /ml and total anti *K. pneumoniae* IgY was found to be 0.522 mg/ml at sixth weeks in the day after immunization.

3.4 Protein Profile by SDS – PAGE

The chicken egg yolk antibodies and its molecular weight was determined by Sodium Dodecyl Sulphate Polyacrylamide gel electrophoresis (SDS- PAGE) using 10% polyacrylamide gel at 100 V and 10 mA according to the method of Laemmli (1970). The SDS- PAGE shows a single band with a molecular weight of 180 kDa in both lane 1 and 2 were compared with marker.

3.5 Estimation of Antibody Titre by ELISA

The antibody titre of each IgY fractions obtained above was determined Indirect ELISA as described by Lee et al. [69]. The antibody titer increases at the time of booster injections, even a minute increase in antibody titer can be traced by this assay. The comparative results show that the antibody titer potencies changes in the courses of immunization. Antibody titre was very low at 0^{th} day egg, then the specific antibody level in the egg yolk was very weak on 21^{st} day and gradually increased and reached the peak on 35^{th} and 49^{th} day. The titre of specific antibody was found to be 1:100000 on 42^{nd} Day and the titre were maintained with booster doses during study period at OD_{490} value

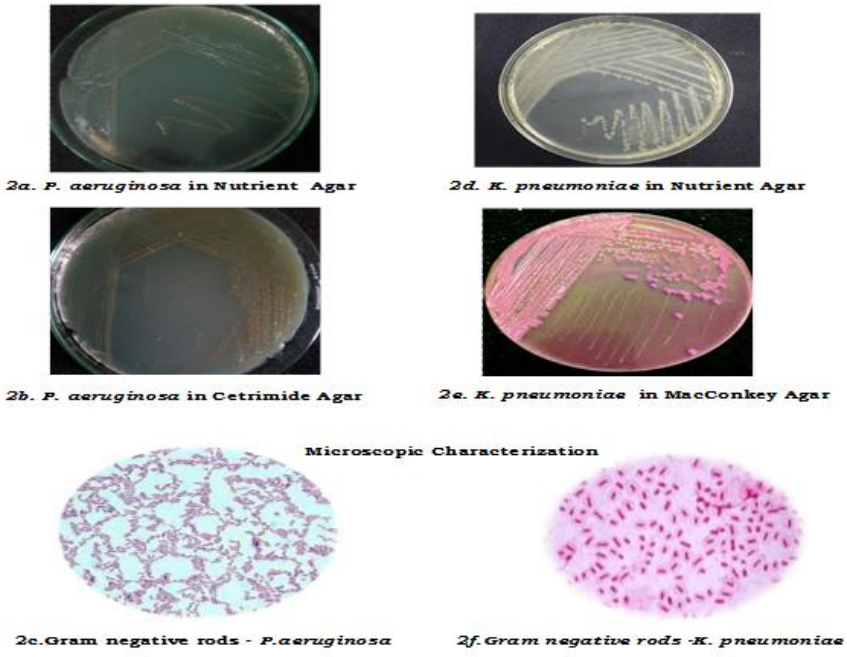

Plate 2. Cultural characterization and microscopic observation of *P. aeuginosa* and *K. pneumoniae*

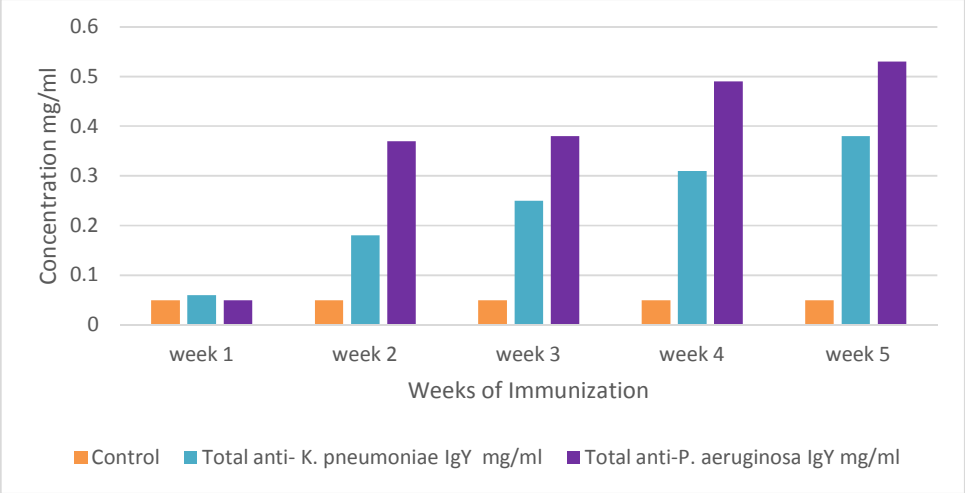

Fig. 1. Estimation of Protein by Lowry *et al., 1951* method

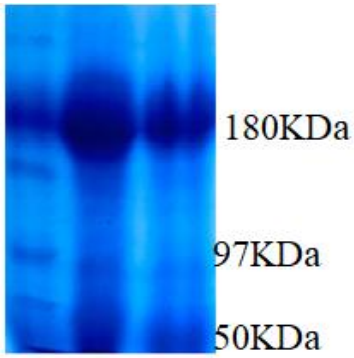

Fig. 2. SDS PAGE (Lane 1 : Marker, Lane 2 : anti- *K.pneumoniae IgY* and anti-*P.aeruginosa IgY)*

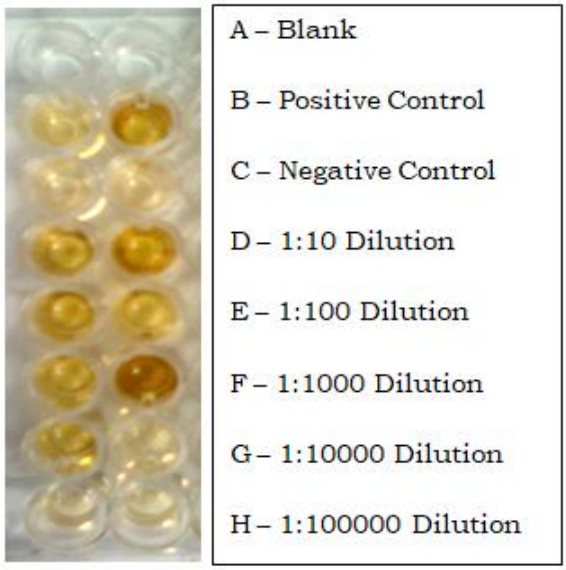

Fig. 3. Estimation of anti- *K.pneumoniae* & anti- *P.aeruginosa* IgY- titration by ELISA

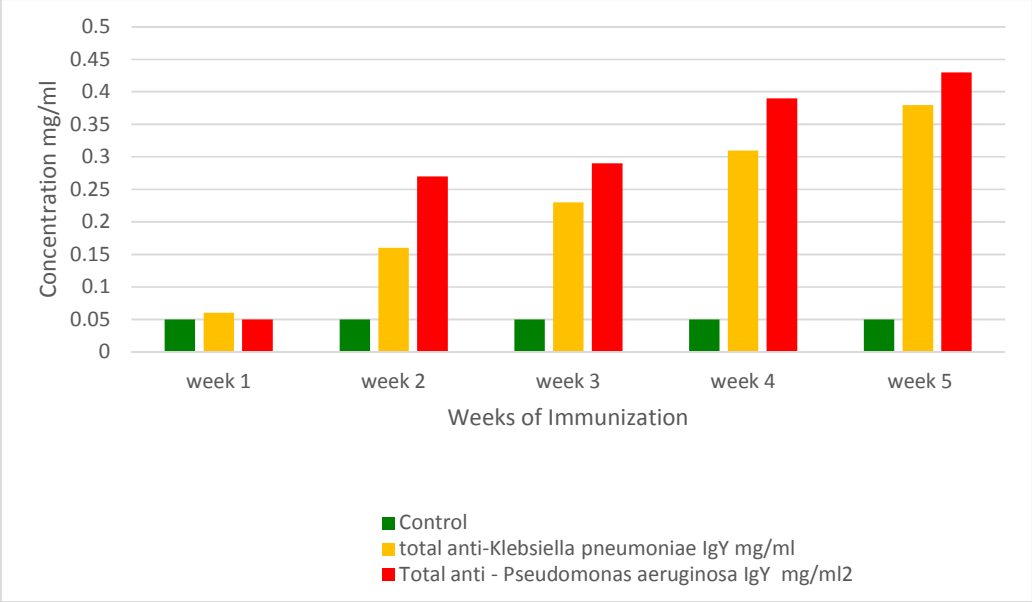

Fig. 4. Concentration of total anti-*K.pneumoniae* IgY and total anti- *P.aeruginosa IgY* antibodies

3.6 Specificity of Antibodies by Growth Inhibition Assay

Growth inhibition assay was performed to check the specific activity of IgY against the bacterial antigens. The growth curves of *K. pneumoniae* and *P.aeruginosa* were plotted for the growth of normal bacterial cells and the growth of bacterial cells with IgY fraction separately. The normal growth curve of based *K.pneumoniae* and *P.aeruginosa* shows as (increasing range) values and specific IgY (20mg/mL) was inhibited the respective pathogens at 4 hours of incubation. The non-specific IgY was inhibited growth was lesser then the specific IgY and the antibiotic was suppressing finally in the time period of 0-2 hours. Based on these results specific IgY was more effective and specific for suppressing the growth of *K.pneumoniae* and *P.aeruginosa.* In which the significant reduction in bacterial growth was observed in the cells incubated with IgY fraction.

Bovine mastitis is an inflammation of the mammary gland. The two major bacterial pathogen, *K.pneumoniae* and *P.aeruginosa* leads to considerable economic losses for the dairy industry (Gill *et al*., 2006). Over 200 different micro-organisms cause mastitis, and these vary greatly in the route by which they reach the cow and in the nature of the disease. At present antibiotics such as penicillin, methicillin, erythromycin etc., are primarily used for the therapy of mastitis. Milk and milk products from the infected cow contain toxins secreted by the microbes and antibiotic residue which may lead to severe health hazards to humans [70-75]. The other demerit associated with the antibiotic therapy is the occurrence of multiple serotypes of organisms inducing the infection, so the vaccines are not very specific for the treatment of disease (Tollersu *et al*., 2001). Furthermore, the increasing prevalence of antibacterial-resistant bacteria has reduced the effectiveness of antibacterial therapy (Guler *et al*., 2005).

Recently, the vaccination via antibodies produced by the vaccinated animal is followed and it acts as another alternative which might be more attractive. This antibody therapy is of great importance [76]. The mammalian antibodies IgG and the chicken egg yolk antibodies came into play a major role in the diagnosis of diseases in poultry and dairy industry [77-83]. Traditionally, the rabbit antibodies (IgG) were used for this purpose. But laboratory production of antibodies involves immunization and bleeding of animals, causing distress to them [84-89]. Egg yolk immunoglobulin can be isolated from the egg yolks of immunized hens by several simple steps without distressing the birds (Akita and Nakai, 1992). IgY can be easily produced and this antibody has received much attention and was

found to efficiently prevent or control pathogen infections in animals [90]. The European Centre for The Validation of Alternative Methods (ECVAM) recommends that Egg yolk immunoglobulins should be used instead of mammalian antibodies for animal welfare reasons [90].

Specific IgY can prevent or control infections caused by *E. coli* in piglets (Jin *et al*., 1998), by *rotavirus* in calves [91] and by *Salmonella* in mice (Gurtler *et al.,* 2004). in dairy cows. Chickens store high contents of IgY in the yolk and are considered to be efficient antibody producers (Gottstein and Hemmeler, 1985). In a period of 6-week, one immunized hen produces 298 g of IgY, which is much higher than the serum antibody (16.6 mg) obtained from one rabbit [92] Moreover, due to the phylogenetic distance between birds and mammal (Jensenius *et al*., 1981), chickens produce more specific antibodies against mammalian antigens than do mammals. The IgY is superior to serum antibody due to higher levels of specific antibodies (Orlans, 1967; Rose *et al*., 1974) and relative ease of purification (Akita and Nakai, 1992) with low cost [68]. The egg yolk antibody has also an advantage over the serum antibody because of its compatibility with modern animal protection regulations (Gottstein and Hemmeler, 1985).

On the basis of the advantages of IgY over the mammalian antibodies entitled in the previous report, the present study focused to develop egg yolk IgY antibodies to control the morbidity and mortality of the bovine mastitis dairy industry from the infection and diseases caused by the predominant bacterial pathogens such as *K.pneumoniae, P.aeruginosa* instead of the treating the infected cattle's using antibiotics [93-99]. The prepared whole cell antigens were used to immunize the 21 weeks old white leghorn chickens to generate IgY. Subsequent booster doses were given at weekly interval to raise the antibody titer in the egg yolk. The eggs were collected, stored and antibodies were purified from chicken egg yolk by Polson et al. [68] method. The molecular weight of the purified IgYs were confirmed as 180kDa through SDS PAGE (Laemmli, 1970). The electrophoretic band pattern obtained in this study was similar to that of the bands obtained by Kaviyarasan *et al*., 2003. The total protein and anti-*P. aeruginosa* IgY concentration was estimated by Lowry *et al.,* 1951. The Total protein concentration of antibody was found to be 8.022 mg /ml and total anti *P. aeruginosa* IgY was found to be 0.511 mg/ml at sixth weeks in the day after immunization. The total protein and anti- *K. pneumoniae* IgY concentration was estimated by Lowry *et al.,* 1951. The Total protein concentration of antibody was found to be 7.997 mg /ml and total anti *K. pneumoniae* IgY was found to be 0.522 mg/ml at sixth weeks in the day after immunization.

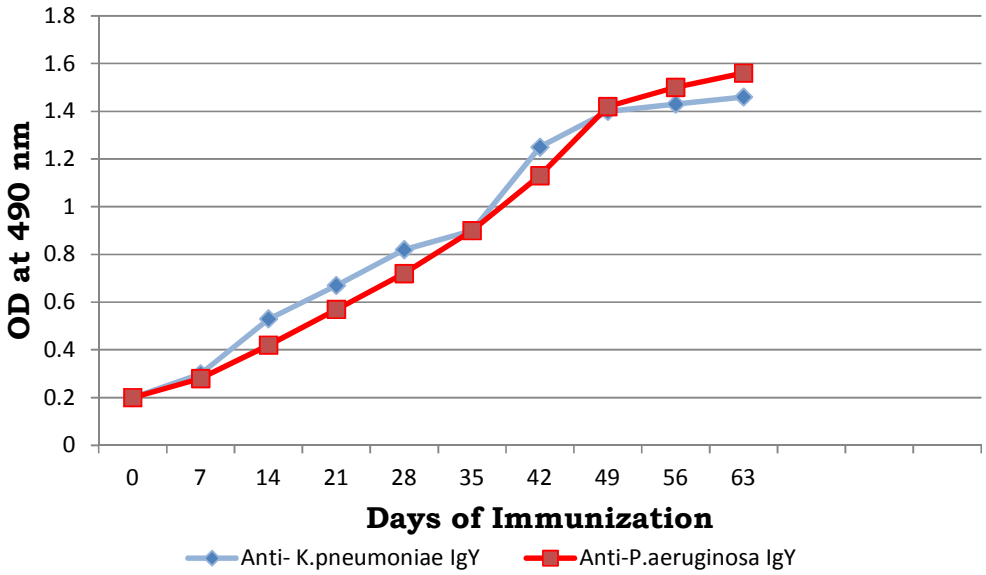

Fig. 5. Growth inhibitory assay graph

The antibody titre of egg yolk antibodies was determined by ELISA [69] showed that the presence of antigen specific antibodies for the specific bacterial pathogens. The specific antibody level in the egg yolk was very weak on 21st day and gradually increased and reached the peak on 35th and 49th day. The titer of specific antibody was found to be 1:100000 on 42nd Day and the titer were maintained with booster doses during study period. It was possibly due to the gradual accumulation of IgY during the yolk formation period by selective active transport (Kitaguchi*et al.,* 2008). The growth inhibition is dose dependents this result is note at the growth decreased with increased specific IgY concentration the mechanism by which antibodies can suppress bacterial growth with particularly components in the bacterial surface which are crucial factors for the bacteria growth may be recognize and bound by related polyclonal antibodies (Hatt *et al*.,1990).

4. CONCLUSION

This study shows that specific IgY antibodies was produced by immunizing hens with a mastitis associated *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* IgY as a complement or alternative to antibiotics offers a possibility to avoid development of antibiotic resistance. Passive immunotherapy with specific IgY may be a promising alternative with high specific nature and low cost effective.

ETHICAL APPROVAL

Animal Ethic committee approval has been taken to carry out this study.

ACKNOWLEDGEMENT

The authors are thankful and gratefully acknowledge to our college funding department DBT-Star scheme, DST-FIST scheme, and to the management of Dr.N.G.P. Arts and Science college, Coimbatore, our college Principal, Deans of Dr.N.G.P. Arts and Science college, Coimbatore as well as all faculty members and our guide, Department of Microbiology, Dr.NGP.Arts and science college, Coimbatore for providing constant support for this entire work. (Communication number: DrNGPASC BS056)

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Akita EM, Nakai S. Comparision of four purification methods for the production of immunoglobulins from eggs laid by hens immunized with an enterotoxigenic *E.coli* strain. J. Immunol. Meth. 1993;160:207-14.
2. Aaristrup FM, Agerso P, Ahrens JCO, Jorgensen M, Madse, Jenson LB. Antimicrobial susceptibility and presence of resistance genes in staphylococci from poultry. Vet. Microbiol. 2000;74:353-64.
3. Adiri RS, Gophna U, Ron EM. Multilocus sequence typing (MLST) of *P. aeruginosa*O78 strains. FEMS Microbiol. Lett. 2003;222:199-203.
4. Allison JR. Antibiotic residues in milk. Br Vet J. 1985;141:9–16.
5. Alterkruse SF, Elvinger Y, Lec KY, Tollyfson LK, Pierson EW, Eifert J, Sriranganathan N. Antimicrobial susceptibilities of Escherichia coli strains from a turkey operation. J. Am. Vet Med. Assoc. 2002;221:411-16.
6. Balaban N, Rasooly A. Staphylococcal enterotoxins. *Int J Food Microbiol*. 2000;61:1-10.
7. Barnes JH, Vaillancourtand N, Gross JP, Saif YM, Barnes HJ, Glisson J, Fadly AM, Mc Donald LR, Swayne DE. (Eds) Colibacillosis Diseases of Poultry. *Lowa state university press*. 2008;631-52.
8. Baron Pier and Ramphal. Fischetti VA, Novick RP, Ferretti JJ. *P. aeruginosa*endrotoxin. (eds) Gram-negative pathogens. ASM Press, Washington DC. 2001;367–78.
9. Barvenich C. Is it possible to predict the severity of acute mastitis during early lactation. Proceeding of Journals National. 1999;43-52.
10. Belschner AP, Hallberg JW, SC. *K. pseudomonas*mastitis therapy revisited. In Proc 35th Annu Mtg Natl Mastitis Council. 1996;116–22.
11. Blanco JE, Blanco M, Mora A. Prevalence of bacterial resistance to quinalones and other antimicrobials among avian *Escherichia coli* strains isolated from septicemic and healthy chickens in spain. J. Clin. Microbiol. 1997;35:2184-85.
12. Gross WB. Disease due to Escherichia coli in Poultry. In: Gyles, CL. (Ed), Escherichia coli in domestic animals and humans. CAB international, Walling ford, UK. 1994;237-59.

13. Halpin-Dohnalek MI, Marth EH. *Staphylococcus aureus*: production of extracellular compounds and behavior in foods. J. Food Prot. 1989;52:267–82.
14. Harmon RJ. Somatic cell count: A primer. In: Annual Meeting National Mastitis Council, 40. Reno. Proceedings. Madison: National Mastitis Council. 2001;3-9.
15. Hatta H. Productivity and some properties of egg yolk antibody (IgY) against human rotavirus compared with rabbit IgG. Biosci. Biotechnol. Biochem. 1993;57(3):292-98.
16. Hatta H, et al. Passive immunization against dental plaque formation in humans: effect of mouth rinse containing egg yolk antibodies (IgY) specific to *Streptococcus* mutans. Caries Res.1997;31:268-74.
17. Heller ED, Smith HW. The incidence of antibiotic resistance and other characteristics amongst *Escherichia coli* strains causing fatal infection in chickens: the utilisation of these characteristics to study the epidemiology of infection. J. Hyg. 1973;71:771-81.
18. Nair MKM, Joy J, Vasudevan P, Hinckley L, Hoagland TA, Venkitanarayanan KS. Antibacterial effect of caprylic acid and monocaprylin on major bacterial mastitis pathogens. J. Dairy Sci. 2005;88:3488–95..
19. Nordhaug ML, Nesse LL, Norcross NL, Gudding R. A field trial with an xperimental vaccine against Staphylococcus aureus mastitis in cattle - Clinical parameters. J. Dairy Sci. 1994;77:1267–75.
20. Nordhaug ML, Nesse LL, Norcross NL, Guddin R. A field trial with an experimental vaccine against *Staphylococcus aureus* mastitis in cattle. 2. Antibody response. J. Dairy Sci. 1994;77:1248–76.
21. Oldham ER, Daley MJ. Lysostaphin: use of a recombinant bactericidal enzyme as a mastitis therapeutic. Characterization of specific egg yolk immunoglobulin (IgY) against mastitis-causing Escherichia coli J. Dairy Sci. 1991;74:4175–4182.
22. Oliver SP, Lewis MJ, Gillespie BE. Influence of prepartum antibiotic therapy on intramammary infections in primigravid heifers during early lactation. J Dairy Sci. 1992;75:406–14.
23. Pereira MS, Leal NC, Leal TC. Typing of human and bovine *Staphylococcus aureus* by RAPD-PCR and ribotyping- PCR. Lett Appl Microbiol. 2002;35:32–6.
24. Prealta RC, Yokoyama H, Ikemori Y, Kuroki M, Kodama Y. Passive immunization against experimental Salmonellosis in Mice by orally administered hen egg yolk antibodies specific for 14-KDa Fimbriae of Salmonella enteriditis J. Med. Microbiol. 1994;41:29-35.
25. Bollen LS, Hau J. 1996. Chicken eggs in polyclonal antibody production. *Scand* J AnimSci. 1996;23:85-91.
26. Bongers JF, Franssen A, Tielen MJ. Antimcrobial resistance of *Escherichia coli* isolates from the faecal flora of veterinarians with different professional specialities. Vet.Q. 1995;17:146-49.
27. Booth JM, Harding F. Testing for antibiotic residues in milk. Vet Rec. 1986;119:565–69.
28. Brooke BE, Frost AJ, Hill AW. Atleast two toxins are involved in Escherichia coli mastitis. Experientia. 1981;37:290-92.
29. Butterworth A. Infectious components of broiler lameness: A review. World's poultry science Journal. 1999;5614:327-52.
30. Chambers HF. The changing epidemiology of *Staphylococcus aureus*? Emerg Infect Dis. 2001;7:178–82 .
31. Chang HM, Ou-Yang RF, Chen YT, Chen CC. Productivity and some properties of immunoglobulin specific against *Streptococcus* mutans srotype C in chicken egg yolk (IgY). *J.* Agric. Food Chem.1999;47:61-6.
32. Clark FD, Watkins SE, Jones FT. Understanding and control of gangrenous dermatitis in poultry houses. Agriculture and Natural resources. University Arkansas cooperative extension service. 2009;44:92-5.
33. Antoniadou A, Kontopidou F, Poulakou G, et al. Colisten-resistant isolates of *Klebsiella pnemoniae* emerging in intensive care unit patients: First report of a multiclonal cluster. J. Antimicrob. Chemother. 2007;59(4):786-90.
34. Crossley KB, Archer GL. The Staphylococci in Human Disease. Churchill Livingstone, New York. 1997;565–82.
35. Devi CM, Bai MV, Lal AV, Umashankar PR, Krishnan LK. An improved method for isolation of anti-viper venom antibodies from chicken egg yolk. *J.* Biochem. Biophys. 2002;51:129–38.
36. Dhanda MR, Sethi MS. Investigation of mastitis in India. ICAR Res. Series No. 35. New Delhi, India; 1962.
37. Dua K. Incidence, etiology and estimated economic losses due to mastitis in Punjab

and in India- An update. Indian Dairyman. 2001;53(10):41-48.

38. Ewers C, JanBen S, KieBhing HC, Phillip A, Wieler LH. Molecular epidemiology of Avian Pathogenic *Escherichia coli* (APEC) isolated from colisepticemiapoultry. Vet. microbiol. 2004;104:91-101.
39. Fioretto F, Cruz C, Largeteau A, Sarli TA. Inactivation of *Staphylococcus aureus* and *Salmonella enteritidis* in tryptic soy broth and caviar samples by high pressure processing. Braz J Med Biol Res. 2005;38:1259–65.
40. Fox LK, Hancock DD. Ecology of *Staphylococcus aureus* isolated from various sites on dairy farms. J Dairy Sci. 1994;77:3354–64.
41. Gassmann M, Thömmes P, Weiser T, Hübscher U. Efficient production of chicken egg antibodies against a conserved mammalian protein. FASEB J. 1990;4:2528-32.
42. Gomis SM, Babiuk DL,Godson B, Allan T, Thrush TH, Ownsend P, WIllson E, Waters R, Hecker H, Potter A . Protection of chickens against *Escherichia coli* infections by DNA containing CPG motifs. Infect. Immun. 2003;71:857-63.
43. Gomis SM, Riddel C, Potter AA, Allan BJ. Phenotypic and genotypic characterization of virulence factors of Escherichia coli associated from broiler chickens with simultaneous occurance of cellulitis and other colibacillosis lesions. Can. J. Vet. Res. 2001;65:1-6.
44. Green MJ, Huxley JN, Bradley AJ. A Rational approach to dry cow Theraphy. Background and Current perspectives. In practice. 2002;24:582-87.
45. Chalgoumi R, Beckers Y, Portetelle D, Thewis A. Hen egg yolk antibodies (IgY), production and use for passive immunization against bacterial enteric infections in chicken: A review. Biotechnol, Agron. Soc. Environ. 2009;13(2):295-308.
46. Lindberg E, Adlerberth I, Wold AE. Antibiotic resistance in Staphylococcus aureus colonizing the intestines of Swedish infants. Clin Microbiol Infect. 2004;10:890–94.
47. Lo´pez HR, Noleto AL, De las Hera MV, Bergdoll MS. Selective terotoxin production in foods by *Staphylococcus aureus* strains that produce more than one enterotoxin. J. Food Prot. 1993;56:538–40.
48. Ma J, Cocchiaro J, Lee JC. Evaluation of serotypes of Staphylococcus aureus strains used in the production of a bovine mastitis bacterin. J. Dairy Sci. 2004;87:178–82.
49. Mahdavi AH, Rahman HR, Nili N, Samie AH. Chicken egg yolk Antibody(IgY) powder against Escherichia coli O78:K80. Journal of Animal and Veterinary Advances. 2010;9(2):366-72.
50. Maiti SK, Sharma S, Neelesh, Awasthy BK. Studies on sub clinical mastitis in cattle and buffaloes of Durg area of Chhattisgarh. Veterinary Practitioner. 2003;4(2):90.
51. Mallikarjunaswamy M.C. and Krishnamurth G.V. 1997. Antibiogram of bacterial pathogens isolated from bovine mastitis cases. *Indian Vet. J.*74(10): 885-87.
52. Marshall MJ, Bohach GA, Boehm DF. Characterization of *Staphylococcus aureus* betatoxin induced leukotoxicity. J Nat Toxins. 2000;9:125–38.
53. McEwen SA, Black WD, Meek AH. Antibiotic residues (bacterial inhibitory substances) in the milk of cows under label and extra-label conditions. Can Vet J. 1992;33:527–34.
54. Miles T, McLaughlin W, Brown PD. Antibacterial resistance of *Escherichia coli* isolates from broiler chickens and humans. BMC Vet. Res. 2006;2:7-9.
55. Mohammed SM. et al. Deposition of genetically engineered human antibodies into the egg yolk of hens. Immunotechnology. 1998;4:115-25.
56. Moreillon P, Que YA, Glauser MP. *Staphylococcus aureus*. In: Mandell GL, Bennett JE, Dolin R (eds) Principles and practice of infectious diseases, 6th edn. Elsevier Churchill Livingstone, Philadelphia. 2005;2321–51.
57. Hensen SM, Pavicic MJ, Lohuis De, Hoog JA, Outrel P. Location of *Staphylococcus aureus* within the experimentally infected Bovine udder and the expression of capsular polysaccharide Type 5 in situ. J. Dairy. Sci. 2000;83:1966-75.
58. Hogan JS, Smith KL, Todhunter DA, Schoenberger PS. Field trial to determine efficacy of an Escherichia coli J5 mastitisvaccine. J. Dairy Sci. 1992;75:78–84.
59. Jablonski LM, Bohach GA. *Staphylococcus aureus*. In: Montville Food Microbiology: Fundamentals and Frontiers, 2nd edn. ASM Press, Washington DC. 2001;410–34.

60. Kariyavasam S, Wilkie BN, Gyles C. Resistance of broiler chickens to *Escherichia coli* respiratory tract infection induced by passively transferred egg-yolk antibodies. Vet. Microbiol 2004;.98: 273-84.
61. Kirk JH, Berry IA, Gardner JM. Dry cow antibiotic treatment in a herd with low contagious mastitis prevalence. In Proc 36th Annu Mtg Natl Mastitis Council. 2002;164.
62. Knezevic P. and Petrovic O. 2008. Antibiotic resistance of commencel*Escherichia coli* of food producing animals from three Vojvodinian farms, Serbia. *Int.J.Antimicrob.Agents*. 31: 360-63.
63. Kwaga JK, Allan J, Van Der Hurk JV, Seida H, Potter AA. Car AB. Mutant of avian pathogenic *Escherichia coli* serogroup O2 is attenuated and effective as a live oral vaccine against colibacillosis in turkeys. Infect. Immun. 1994;62:3766-72.
64. La Ragione RM, Narbad A, Gasson MJ, Woodword MJ. *Invitro*charecterisaton of *Lactobacillus johnsonii*.FI9785 for use as a defined competitive exclusion agent against bacterial pathogens in poultry. Lett. Appl. Microbiol. 2004;38:197-205.
65. Lampang KN, Chongsuvivatwong V, Teanpais R, Kitikoon V. Concordance of antibiotic resistance and ERIC-PCR DNA fingerprint pattern in *Escherichia coli* isolated from farmer and broiler in the same farm. World. J. Microbiol. Biotechnol. 2008;24:1741-46.
66. Eric LJ, Carolyn LM. Staphylococcus infection in broiler breeders. In proceedings of avian skeletal disease symposium. AAAP/AVMA, San Antonio, Tx. 1990;83-85.
67. Sudhan NA, Singh R, Singh M, Soodan JS. Studies on prevalence, etiology and diagnosis of sub clinical mastitis among cross bred cows. Indian J. Anim. Res. 2005;39(2):127-30.
68. Polson A, Von Wechmar MB, Van Regenmortel MHV. Isolation of viral IgY antibodies from egg yolks of immunized hens. Immunol. Commun. 1980;9:475.
69. Lee EN, Sunwoo HH, Menninen K, Sim JS. *In vitro* studies of chicken egg yolk antibody (IgY) against *Salmonella* Enteritidis and *Salmonella* typhimurium. Poult. Sci. 2002;81:632-41.
70. Tini M. Generation and application of chicken egg-yolk antibodies. Com. Biochem. Physiol. 2002;131:569- 74.
71. Tompkin RB. Indicator organisms in meat and poultry products. Food Technol. 1983;37: 107-10.
72. Vandekerchove DP, De-Herdt P, Laevens H, Pasmans F. Coolibacillosis in caged layer hens: charecteristics of the disease and the aetiological agent. Avian Pathol. 2004;33:117-25.
73. Wray C, Corkish JD. Resistance of broiler chicken to *Escherichia coli* respiratory tract infection induced by passively transferred egg-yolk antibodies. J. Veterinary *microbiology*. 1997;98:273-84.
74. Yu-Hong Z, Li-ji J, Jie G, Xiao-Yu L, Ya-NanLu J, Yong-Ping X. Characterisation of specific egg yolk immunoglobulin (IgY) against mastitis-causing Escherichia coli. Vet. Microbiol. 2007;98:273-84.
75. Nordberg P, Monet DL, Cars O. Background document for WHO project: Priority medicines for Europe and the world. "A Public health approach to innovation; 2004.
Available: http://mednet3.who.int/priority needs/
76. Coleman C, Marilyn A. Oral administration of chicken yolk immunoglobulins to lower somatic cell count in the milk of lactating ruminants. US Patent. 1996;5:585-98.
77. Radostits OM, Gay CC, Blood DC, Hinchcliff KW. Mastitis. In: Veterinary Medicine, 9th ed., W.B. Saunders Company Ltd, London. 2000;603-87.
78. Ragunath D. Emerging antibiotic resistance in bacteria with special reference to India. J. biosci. 2008;33(4):593-603.
79. Rahimi S, Shiraz ZM, Salehi TZ, Mohammad A, Karimi T, Jesse L. Prevention of *Salmonella* infection in poultry by specific egg derived antibody. In. Jn. of. Poult. Sci. 2007;6(4):230-35.
80. RobersNickerson SC, Owens WE, Boddie RL. Mastitis in dairy heifers: initial studies on prevalence and control. J Dairy Sci . 1995;78:1607–18.
81. Roghmann M, Taylor KL, Gupte A. Epidemiology of capsular and surface polysaccharide in *Staphylococcus aureus* infections complicated by bacteraemia. JHosp Infect. 2005;59:27–32.
82. Schade R, Staak C, Erhard M, Hugel MH. The production of avian antibodies: IgY. The report and recommendations of

ECVAM workshop 21. ATLA. 1996;24:925-34.

83. Shahab Quershi, Stuart Bronze. The real threat of Klebsiella pneumonia carbapnemase-producing bacteria. Lancet Infect Dis.2014 Apr.9(4):228-26
84. Sharma Neelesh. Foot and Mouth Disease - Mastitis Cascade in Dairy Cattle: A field study. International Journal of Zoological Research. 2008;4(1):64-67.
85. Sharma Neelesh, Pandey V, Upadhya SR. Recent trends in the diagnosis of bovine mastitis under field condition. Indian Dairyman. 2009;61(4):21-7.
86. Sharma A, Dhingra P, Pander BL, Kumar R. Bovine subclinical mastitis: prevalence and treatment with homeopathic medicine. Intl. J. Cow Sci. 2006;2:1-12.
87. Sharma N, Maiti SK, Sharma KK. Prevalence, etiology and antibiogram of microorganisms associated with sub clinical mastitis in buffaloes in Durg, Chhattisgrh State (India). International J. Dairy Sci. 2007;2(2):145-51.
88. Sherman DM, Acres SD, Sadowski PL, Springer JA. Bray B, Raybould TJG. et al. Protection of calves against fatel enteric colibacillsis by orally administrated Escherichia coli K99-specific monoclonal antibody. Infect.Immun. 1983;653-58.
89. Shimizu M. Molecular stability of chicken and rabbit immunoglobulins G. Biosci. Biotechnol. Biochem. 1992;56:270-74.
90. Carlander D, Kollberg H, Wejaker P, Larsson A. Peroral immunotheraphy with yolk antibodies for the prevention and treatment of enteric infections. *Immunol. Res.* 2002;21:1–6.
91. Kuroki M. et al. Passive protection against bovine rotavirus in calves by specific immunoglobulins from chicken egg yolk. Arch. Virol. 1994;138:143-48.
92. Shimizu M, Nakai S, Fitzsimmons RC. An-*E. coli* immunoglobulin Y isolated from egg yolk of immunized chickens as a potential food igredient. J. Food Sci. 1988;5:1360-66.
93. Shpige NY, Levin D, Winkler M, Saran A, Ziv G, Bottner A. Efficacy of quinine for the treatment of cows with mastitis experimentally induced using E. coli. J. Dairy. Sci. 1997;80:318-23.
94. Singh R, Sharma-Neelesh S, Soodan JS, Sudhan NA. Etiology and sensitivity of bacterial isolates from sub clinical mastitis in cattle from Jammu region. Journal of Research SKUAST-J.2005;4(2):223-24.
95. Skrabanja AT, De Meere AL, De Ruiter RA, Van DO. Lyophilization of biotechnology products. PDA J. Pharm. Sci. Technol. 1994;48(6):311-17.
96. Skrzypek R., Wtowski J. and Fahr R.D. Factors affecting somatic cell count in cow bulk tank milk - a case study from Poland. *J. Vet. Med. A, Physiol. Pathol. Clin. Med.*;201-09.
97. Sol J., Sampimon O.C. and Snoep J.J. 1997. Factors associated with bacteriological cure during lactation after therapy for subclinical mastitis caused by*Pseudomonas aeruginosa*. *J Dairy Sci*. 80: 2803–08.
98. Songer JG, Post KW. Veterinary Microbiology. Bacterial and Fungal agents of animal diseases. Suggestion for their taxonomic classification. Balcp. Hyg. I. Abt. Orig. A. 2005;217:176-82.
99. Sunwoo HH. Growth inhibitory effect of chicken egg yolk antibody (IgY) on Escherichia coli O157. J. Food Sci. 2002;75:342-34.

Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sdiarticle4.com/review-history/67285