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GENERATION AND CHARACTERIZATION OF CHICKEN EGG YOLK ANTIBODIES (IGY) AGAINST CLOSTRIDIUM DIFFICILE AND EVALUATING ITS INVITRO NEUTRALIZATION EFFICACY

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ABSTRACT

Passive immunization by oral administration of specific antibodies has been an attractive approach against gastrointestinal tract (GIT) pathogens in both humans and animals. Recently, laying chickens have attracted considerable attention as an alternative source of antibodies for the prevention and treatment of infectious GIT diseases. Chicken egg yolk antibodies (IgY) were raised in twenty two weeks old white leghorn chicken against formalin killed whole cell antigen of *Clostridium difficile* (ATCC 9689). The level of the antibody in serum was monitored and booster doses were given wherever necessary. The antibodies were purified from the egg yolk of immunized chicken using PEG and Ammonium Sulphate precipitation method and further purified by dialysis method. The protein profile of anti *Clostridium difficile* IgY antibodies were analyzed by SDS-PAGEand Quantitative Titration of IgYantibodies by ELISA. High titer of specific antibody was found to be 1: 100000 on 91stday detected by modified ELISA and the titer were maintained with booster doses. The protein concentration of the egg yolk was 39.99 ± 0.79 mg/ml and the total IgY concentration in egg yolk was increased during the immunization period and reached maximum of 15.26± 0.57 mg/ml. *In-vitro* efficacy of anti-*C.difficile* IgY was determined by growth inhibition assay. A significant reduction in the growth was observed after 16 hours of incubation. These

findings may open the door for significant advances in IgY technology and passive immunotherapy with an alternative high specific nature and low cost effective.

KEYWORDS: Gastrointestinal tract (GIT), Chicken egg yolk antibodies (IgY), polyethylene glycol (PEG), Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE), Enzyme Linked Immuno Assay (ELISA).

INTRODUCTION

Clostridium difficile is an anaerobic spore forming bacillus widely distributed in nature and is particularly prevalent in hospitals. Conventional antibiotics alter both the numbers and complexity of the normal gastrointestinal (GI) micro flora; thereby reducing the ability of these protective flora to competitively exclude opportunistic from colonizing the GI tract. This may render hospitalized patients susceptible to colonization by *C.difficile.* [1] *C.difficile* isolates that are capable of producing toxins A and B are regarded as toxigenic. The clinical features typically include diarrhea, lower abdominal pain and systemic symptoms such as fever, anorexia, nausea and malaise but they can range from mild diarrhea to pseudomembraneous colitis, toxic mega colon and multi-system organ failure, which may be fatal. [2] *C.difficile* –associated diarrhea (CDAD) is an important infection control issue. In Taiwan a retrospective study shows the incidence of *C difficile* infection was 42.6 cases per 100,000 patient days and was increasing in recent years. [3] Advanced age, the use of antibiotic, prolonged hospitalization, the presence of co-morbidity with functional impairment or immune gene polymorphism is associated with increased rates of *C.difficile* infection and disease recurrence. [4, 5] The interaction of *C.difficile* with the innate immune system likely plays a major role in the pathogenesis of colitis or pseudo membranous disease.

Toxigenic *C difficile* colonization (tcdc) has been reported as an independent factor of subsequent development of CDAD. [6] In recent years the incidence of *C.difficile* associated diarrhea (CDAD) has risen dramatically due to the frequent use of board spectrum antibiotics, especially in North America and Europe. [7, 8] The accepted approach to treating CDI is to replace the antibiotic responsible for initiating the condition with metronidazole, is sensitive to *C.difficile.* 80% of cases, this approach lead to complete recovery without further complications. In some cases recurring CDI (rCDI) can occur, due to a failure of the normal gastrointestinal flora to become re-established after the Metronidazole is discontinued and leads to *C.difficile* relapse or reinfection. Multiple recurrences are not unusual, resulting in a significant reduction in the quality of life in these persons. In response to the increasing risk

of CDI, to human health, much research has focused on new and improved treatment regimens, particularly persons suffering from rCDI. These studies have concentrated on novel toxin neutralizing agents, antibiotics which are more sparing of the normal GI micro flora, and pre or probiotic approaches (Kelly *et al*., 1996; Kink & Williams, 1998; Aboudola *et al*., 2003; Giannasca & Warny, 2004; McFarland, 2009; Merrigan *et al*., 2009; Mondan *et al*., 2009). There are indications that *C.difficile* was colonizing the mammalian GI tract. For example, persons asymptomatically colonized by *C.difficile* display a decreased risk of developing CDI and pre-colonizing clindamycin treated hamsters with non-toxigenic *C.difficile* strains protected the animals from a subsequent challenge with a wild-type toxigenic strain (Sambol *et al*., 2002; Merrigan *et al*., 2003). A wide range of alternatives to antibiotics have emerged like (organic acids, probiotics, herbal extracts and others), of which passive immunization with antibodies is an approach with great potential due to its high specificity (Sol *et al*., 1997).Passive immunization is basic to human and animal health. One way is the use of antibodies (Doyle 2001). The ability of antibodies to bind antigen with a high degree of affinity and specificity has led to their ubiquitous use in a variety of resources (Berghman *et al*., 2005). Their use in diagnostic assays and as therapeutics had a profound impact on improving the health and welfare of both humans and animals. However, the method has associated problems in terms of cost and productivity. Recently, the production of IgY antibodies from the eggs of chicken which were immunized with pathogens has been the focus of attention in immunotherapy and diagnosis since avian can actively transper immunoglobin to the egg yolk at the same concentration levels as in mammals (Sunwoo *et al*., 2002; Buchwald and Profski 2003).

The use of chicken antibody on a large scale offers several advantages, such as low costs, large yield and scale able production and storability. The efficacy of IgY has been demonstrated in several applications, for example the treatment and prevention of fatal enteric coliform bacillus in neonatal piglets and calves, viral diarrhea in infants, dental caries, canine parvovirus, and snake venom (Mini and Kovas- Nolan 2002; Schade *et al*., 2005; Shin *et al*., 2002). Despite all the advantages offered, the IgY obtained from the egg yolks of immunized hens need to be better evaluated. IgY used in human medicine has been found be effective against a number of human pathogens and diseases both in vitro and in laboratory animal studies and clinical settings. One of the most successful clinical applications of IgY has been in the prevention of Pseudomonas aeruginosa (Pa) colonization in the airways of cystic fibrosis (CF) patients. In 2008, Orphan drug designation was granted for IgY antibody

against (Pa) for the treatment of (CF) in humans by the European Medicines Agency. In ongoing trials in (CF) patients, a mouth rinse containing purified anti-Pa IgY given a continuous basis could significantly reduce or prevent (Pa) colonization there by reducing the need for antibodies (Carlander *et al*., 2000; Kollberg *et al*., 2003; Nilson *et al* ., 2003, 2008). These studies have shown that specific IgY is effective for immunotherapy for long treatment periods without any side effects (Wilson *et al*., 2007). Another clinical application of IgY in humans is for the prevention of Helicobacter pylori infection. Likewise, a number of studies and case reports have indicated that passive immunotherapy is a successful therapy for patients suffering chronic relapsing *C.difficile* infection, which did not respond to standard treatment (i.e. ., antibiotic therapy). Here in this study we contend that colonizing factor specific IgY preparation, either alone or in combination with other therapeutic strategies, depict for treating CDI because they can be mass-produced inexpensively, and they are listed in a safe category for human use. There is also much evidence that purified IgG (Kelly *et al*., 1996) and IgY (Kenk & Williams, 1998) antibodies retain their inhibitory action after passing through the stomach and small intestine of many mammals. Colonizing factor – specific IgY preparations should will facilitate eliminating *C.difficile* from the intestines of CDI, the infected persons while at the same time, sparing the normal GI microflora and consequently reduce incidence of (rCDI).This study therefore provides a simple and efficient way of producing IgY antibodies against *C.difficile*. Chicken anti *C.difficile* IgY was generated in immunized white leghorn hens using Freund‟s complete adjuvant for primary immunization and Freund‟s in-complete adjuvant for booster immunization. In vitro experiments showed that the immunoglobin from the egg yolk interfered with bacterial growth. These findings indicate that the eggs of hens immunized with appropriate antigen have potential use as a source of passive immunity. Therefore, the objective of this study was to investigate the anti bacterial properties of IgY against *C.difficile* for its specificity and activity by using various in-vitro methods such as ELISA, SDA-PAGE, growth inhibition assay and microscopy.

Antibodies available for research, diagnostic and therapies are mostly monoclonal or polyclonal antibodies. Traditionally, bigger animals such as horses, sheep, pigs, rabbits and guinea pigs were used for the production of polyclonal antibodies, while mice and rats were used for the production of monoclonal antibodies. Nowadays, most frequently chosen mammals for polyclonal and monoclonal antibody production are rabbit and mice respectively. Both technologies have their advantages but also disadvantages. Major problem of monoclonal antibody production is that some antigens from mammalian blood has been

found to be low yielding and laborious in many cases. Both technologies also involve some steps each of which causes distress to the animals involved (i) the immunization itself (ii) collecting of blood samples and (iii) bleeding, which are a prerequisite for antibody preparation. [9] During the past 23 years, the use of chickens instead of mammals for antibody production has increased. 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. In addition, the antibody productivity of an egg laying hen is much greater than that of a similar sized mammal. [10]

Purification of immunoglobulin from mammalian blood is time consuming and expensive. Nowadays, chicken are recognized as a convenient and inexpensive source of antibodies. It has been reported that the amount of immunoglobulin that can be yielded from one egg of an immunized hen is as much as that can be obtained from 300ml of rabbit blood. Chicken egg yolk antibodies [IgY] have been applied successfully for scientific, diagnostic, prophylactic and therapeutic purposes. Because of the phylogenic distance between birds and mammals, mammalian proteins are often more immunogenic in birds than in other mammals and antibody synthesis readily stimulated in hens. [11] However, use of chickens for the purpose offers two advantages. The use of chicken egg for the antibody production, as opposed to mammals represents both a refinement and reduction in animal use. It is a refinement in that the second painful step, the collection of blood is replaced by antibody extraction from egg yolk. It enables the reduction in the number of animals. Larson *et al*. found that IgY is more highly concentrated in egg yolk than it is in serum. [12] The chicken is an excellent producer of antibodies, but despite this, is still an under used resource. This may be due to lack of information concerning the different methods and application where IgY is more advantages compared to the traditional mammalian IgG antibodies. The aim of this work was to use the above approach for generation and preliminary characterization of polyclonal, mono-specific antibodies against the infection producing *C.difficile*.

MATERIALS AND METHODS

Experimental Animal: Twenty oneweeks old White Leghorn chickens in good health condition were obtained from L.K poultry, Palladam. The chickens were maintained in our animal facility with normalfeeding and used for the studyfor the production of anti-*Clostridium difficile*antibodies (IgY)without sacrificing it (Fig: 1).

Fig: 1 -Experimental animals

Bacterial strain: Bacterial strain used for the present study is *Clostridium difficile.* The standard strain was obtained from ATCC(NO: 9689).The strainwas cultured anaerobically on blood agar plates with repeated subculturing andstored for further analysis. Forall these experiments, standardaseptic techniques were followed; includingsterilization of media, glass waresetc.

Preparation of whole cell antigen: The whole cell antigen was prepared under standard indigenous conditions. Pure isolated colonies of *Clostridium difficile*was inoculated in 5 ml of Brain Heart Infusion broth (BHI) in screw cap tubes and incubated at 37º C for 48 hours. Cells were harvested by centrifugation at 7000 rpm for 15-20 minutes. Supernatant was discarded and the pellet was washed three times with phosphate-buffered saline (PBS,pH7.4). After the last wash, pellets were re-suspended in PBS containing 1% (Vol/Vol) formaldehyde and incubated overnight. Excess formaldehyde wasremoved by three washes with PBS. The formalinized *Clostridium difficile*cell suspension was stored at 4º C.

Purity testing of antigen: Complete killing of the bacteria was tested by re-suspending an aliquot of the cell pellet in PBS saline and plating 100µl of this suspension into blood agar medium. The plates were incubated anaerobically at 37º C for 48 hours and examined for the presence of bacterial growth.

Preparation of antigen –adjuvant mixture for immunization: For immunization, *Clostridium difficile*whole cell antigen was mixed homogeneously with an equal volume of adjuvant. This mixing was done by taking the antigen (0.5ml) and adjuvant (0.5ml) in a separate sterile 2ml syringes. The sterile 2ml syringes were connected to each other with a

tube. Then the syringes were pressed to create pressure there by mixing the contents. The antigen –adjuvant emulsion would appear milky white if mixing was proper (Fig: 2).The adjuvant used was Freund's complete adjuvant(FCA)for primary immunization and Freund's in-complete adjuvant(FIA)for booster immunization.

Fig: 2 Adjuvant and Antigen mixing

Generation of antibodies against *C.difficile*in Chickens: Antigen prepared was diluted using sterile saline and adjusted to McFarland Barium Sulphate standard tube no.1. The 'in use' suspension with adjusted capacity shows a final cell concentration of 3 × 108cells. For first immunization, the 22 weeks old white leghorn chickens were intramuscularly injected at multiple sites of the breast muscles with antigen emulsified in FCA(Fig:3a)).After two weeks interval the chickens were immunized with same quantity of antigen emulsified in FIA. Further booster doses were carried out by injecting plain antigen, whereby the chicken was hyper immunized. Blood was sampled at intervals of two weeks from the initiation of the immunization and checked for the presence of anti *C. difficile*antibodies.(Table 1)Further, eggs laid by the chicken under the test were collected regularly and stored at4ºC.

Table: 1 Immunization Schedule

Dosage	Days	Antigen dose	Adjuvant used
Primary Dose	0	$3x10^8$ Cells/ml	FCA
II Dose	14	$3x10^8$ Cells/ml	FIA
III Dose	28	$3x10^8$ Cells/ml	FIA
Booster Dose	**14 Days interval**		

FCA- Freund's complete adjuvant

FIA – Freund's incomplete adjuvant

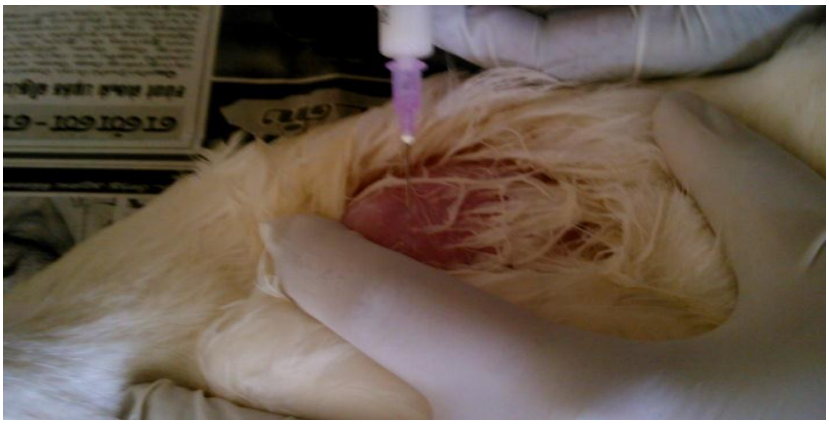

Fig: 3 a)Immunization

Purification and characterization of anti-*C.difficile* Antibodies from Egg Yolk:

Separation of Egg-Yolk: Under strict aseptic techniques the egg yolk was separated from white and was washed with water to remove as much albumin possible. The yolk was rolled on tissue paper and the yolk membrane was punctured using an applicator sticks. Allow the yolk without membrane was allowed to flow into a graduated cylinder. The yolk membrane and any remaining egg white will stick to the tissue paper. The yolk sac was discarded. The amount of yolk obtained was measured. Approximately 10-15ml of yolk obtained from an average sized egg.

Purification of IgY by *Polson et al.*, (1980) method: The egg yolk antibodies were purified according to the method described by Polson *et al*., (1980). [13] Separated yolk from the eggs of immunized or non-immunized hens, were mixed with twice the volume of 100mM Phosphate buffered saline (PBS; pH7.2) and mixed thoroughly. 3.5% w/v polyethylene glycol (PEG 6000) was added and mixed until the PEG completely dissolved. The sample was centrifuged at 10,000 rpm for 20 minutes at 4^0C. A cotton wool (absorbent-type) was firmly placed at the base of the funnel, and the supernatant was filtered through it. The lipid fraction is trapped by the cotton wool. The filtrate must be clear yellow liquid and not milky in appearance; if the filtrate not clear filtration method is repeated. The filtrate volume is recorded, and the PEG concentration was increased (i.e. add 8.5%). The PEG was dissolved completely by mixing. The suspension was centrifuged as on above. Remaining supernatant was discarded and the pellet was dissolved in 10ml PBS and 1.2g and mixed well, then again centrifuged as on above. The pellet was dissolved in 800µl Phosphate Buffer Saline. Then the content was desalted by dialysis process (Fig: 3 b)). The pellet was redissolved in buffer S to the original yolk volume and the equal volume of 4 M ammonium sulphate of pH 7 was

added and incubated at 0ºC for 30 minutes so that antibodies get saturated. The precipitate was centrifuge at 11000 rpm for 20 minutes and the pellet was redissolved in buffer S without NaCl for overnight.

Fig: 3 b) Dialysis and PEG Extraction Method

Determination of protein content in IgY fraction by Lowry *et al.*, (1951): The total protein content was estimated by the method described by Lowry *et al.*, (1951). [14] A quantity of 10mg Bovine Serum Albumin (BSA) was dissolved in 10ml of distilled water and used as Protein stock solution. To a series of clean test tubes 0.2 – 1.0 ml of BSA (Protein stock solution) was added and made up to final concentration of 5 ml with distilled water. From these dilutions 0.2ml was taken in to different test tubes and 2ml of Alkaline Copper Sulphate solution was added to each test tube and incubated for 10 minutes at room temperature. After incubation, 2ml of Folin-Ciocalteau reagent was added to each tube and incubated under dark condition for 30 minutes at room temperature. 10mg of Lyophilized IgY powders from each preparation (Polson *et al.*, 1980 and Water dilution Method (WDM)) were taken and emulsified with 1ml of distilled water in separate tubes and the above procedure was repeated to estimate the total protein content. Finally the optical density (OD) was measured at 660nm. The OD values of IgY powders were compared with standard graph.

Specificity of IgY

The specificity of anti-*Clostridium difficile* antibodies of the chicken serum and egg yolk was determined by Rapid Slide Agglutination Test (RSA). Test was done on a plastic strip; 20µl of antigen and 20µl of IgY were placed and mixed thoroughly by stirring with the help of applicator stick. Then the slide was observed for the appearance of agglutination with in

2minutes. The presence of clumping indicated the agglutination reaction, which confirms the presence of specific IgY antibodies against specific antigen. [Fig: 4 a)]

Protein profiling by SDS-PAGE: The protein profile of anti *Clostridium difficile* IgY antibodies were analyzed by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) as described by Laemmli (1970). [15] According to Laemmli 1970 the proteins are resolved with 10% (W/V) polyacrylamide separating gel and 4% (W/V) polyacrylamide stacking gel at 250 V and 10mA. Equal ratio *Clostridium difficile* IgY antibodies (20µl) and sample treating buffer (20 µl) were mixed well and loaded into sample wells. A wide range molecular weight (6.5–205 KD) marker was also run along with the sample. The samples were run until they reach the bottom of the gel. The characteristic protein pattern for the specific antibodies can be visualized after staining with Coomassie Brilliant Blue (CBB – R 250). The bands can be visualized clearly after destaining it with 7% acetic acid. This removes the dye that was not bound to proteins. The destainer was changed frequently until the background of the gel became colorless.

Quantitative Titration of Antibodies by ELISA: The ELISA procedure adapted was a modified method of the original ELISA procedure described by Gupta *et al*., (1992). Polyvinyl micro titration plates were coated with 100µl of the *Clostridium difficile* antigen that was diluted with carbonate buffer pH 9.0 and incubated at 37º C overnight. After coating the plates were washed with PBS containing 0.05% Tween 20 (PBST) and nonspecific binding sites blocked by adding 200 µl per well of 1% bovine serum albumin in PBS and incubating the plates at 37º C for 1 hour. Plates were subsequently washed with PBST and incubated with 100µl of egg yolk antibodies at appropriate dilutions. Control wells had PBST and pre immune sera served as respective controls. Plates were incubated for one hour at 37º C and subsequently washed with PBST.

For the chicken antibodies 100µl of rabbit anti chicken immunoglobulin coupled to horse raddish peroxidise was added at the appropriate dilutions and plates incubated for 1 hour at 37º C. After incubation the plates were washed with PBST and enzyme activity determined by adding 100µl of freshly prepared substrate solution (4 mg of O-Phenylene diamine dissolved in 10 ml of 50 mm citrate buffer, pH 5.0 containing 10µl hydrogen peroxide). And the plates were allowed to stand at room temperature (dark condition) for 15 minutes. The reaction was stopped by adding 50 µl of 4N H_2SO_4 and plates were read at 490nm in an

ELISA reader. The ELISA titer was defined as the dilution giving at 490nm of 0.2. Crude IgY from non-immunized hens was used as control.

Determination of Physicochemical Properties & Stability of IgY

The stability of IgY in different physicochemical conditions was determined by the methods prescribed by Fan *et al*., (2009).Like i) Heat stability ii) pH stability iii) Pepsin stability of IgY iv) Stability of IgY in Liquid Yolk. The stability of IgY when incubated at different conditions was assessed where the IgY retained its stability at different temperatures, pH, pepsin to IgY did not reduce its activity, when compared to the untreated control.

In-vitro neutralization efficacy of *C difficile* specific IgY by Growth Inhibition Assay

The *C. difficile* culture (ATCC 9689) maintained in 80°C was revived. One loop full of sample was inoculated in BHI agar with 5% sheep blood at 37°C for 3-7 days at anaerobic conditions. The culture was confirmed to be *C. difficile* by cultural and biochemical characteristics. The pure culture identified was transferred aseptically to the filtered BHI broth supplemented with 5% horse serum and allowed to grow for 3 to 4 days under anaerobic conditions (80% N2, 15% CO2, and 5% O2) at 37°C. The cells of bacteria were taken as 1.0 optical density corresponding to 10^8CFU/ml at 600nm in UV/Visible spectrophotometer. The immunized egg yolk against *C. difficile* were purified by water dilution method and freeze dried to obtain IgY powder. 100µl of the stock culture *C. difficile* was mixed with 5ml of BHI broth and incubated at 37°C under anaerobic conditions and the growth curve was studied. A loop full of culture was inoculated in BHI broth and mixed along with *C. difficile* specific and non specific IgY. The samples were incubation overnight at 37°C and the growth inhibition was determined for 48hrs in terms of OD for each time interval of 4 hours at 600 nm using a UV/Visible spectrophotometer.

ETHICAL CONSIDERATIONS: The Institutional Animal Ethics Committee was approved this research work in accordance with "Principles of Laboratory Animal Care." the consent was obtained.

RESULTS & DISCUSSION

(i) Generation and Characterization of the C. difficile IgY

***C. difficile* whole cell Antigen:** The present study was focused to develop egg yolk antibodies against the whole cell antigen of *C. difficile*. For this purpose whole cell antigens were prepared from the Standard strains of C. difficile obtained from American type culture

collection (ATCC # 9689) was revived as per the protocol also confirmed morphologically and biochemically.

Generation of Antibodies in Chickens: The prepared whole cell antigens were used to immunize the 22 weeks old white leghorn chickens to generate IgY. Booster doses were given at 14 days interval to raise the antibody titer in the egg yolk. The pre-immune sera and hyper immune sera were collected at specific time intervals during and after the various immunization schedules. The presence of antibody in chicken serum was assessed by the slide agglutination method.

Egg Collection and Storage: After immunization the eggs were collected and stored at 4°C with proper marking of appropriate antigen name and date to avoid confusion. Then the stored eggs were used for the purification of antibodies from yolks.

Purification of IgY by Polson *et al.*, (1980) method: The method used for purification of chicken egg yolk antibodies were Poly Ethanol Glycol method described by Polson *et al.*, 1980. After dialysis the IgY solution was lyophilized for further studies.

Protein Estimation: Protein content of freeze dried IgY antibodies was estimated by the method described by Lowry *et al.* (1951) using Folin-Ciocalteau reagent. The optical density of the BSA standard was used to plot the graph and the total protein concentration of the IgY powders obtained by Polson *et al.*, method were detected 39.99 ± 0.79 mg/ ml and 15.26 ± 0.57 mg/ml for *C. difficile* (Fig: 5)

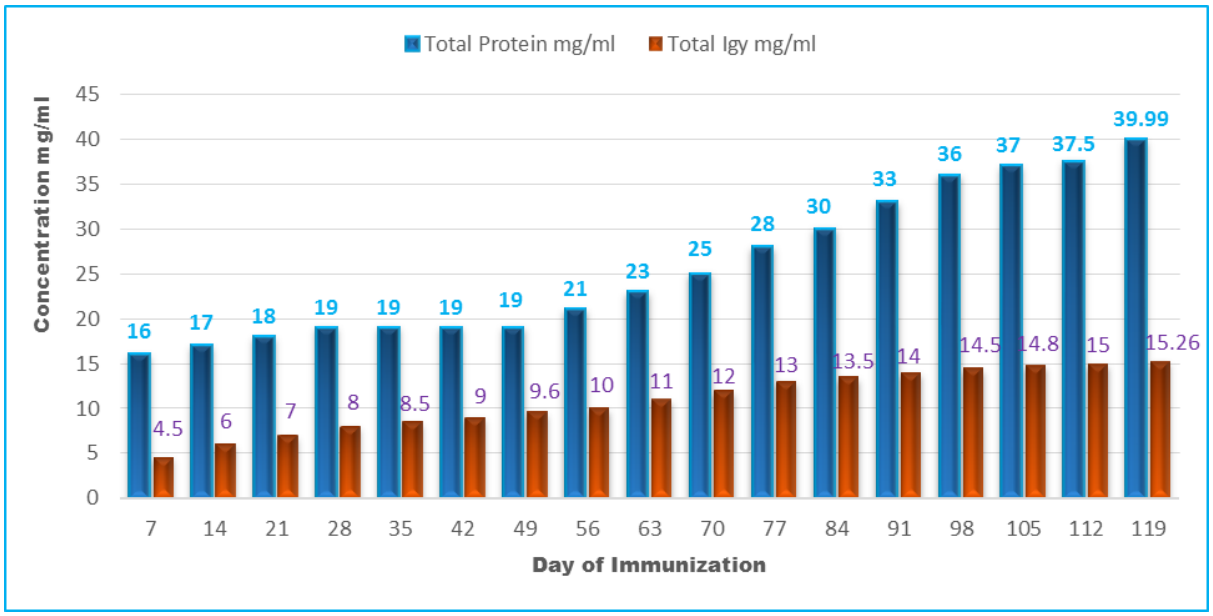

Figure 5: Protein and total IgY concentration in anti- *C. difficile* IgY extract

Determination of Specific Antibodies in yolk and Serum: Specificity of anti-*C. difficile* antibodies in the serum and egg yolk from immunized laying chickens was determined by Rapid slide agglutination Test (RSA). Appearance of agglutination within 2minutes, when the antigen was mixed with the corresponding IgY on plastic strip, revealed that the antibody generated in the chicken serum and the purified IgY-extracts from eggs of immunized Chicken were specific against to their respective antigens[Fig: 4 a)]. With this qualitative determination further titration of the specific IgY could be performed by ELISA.

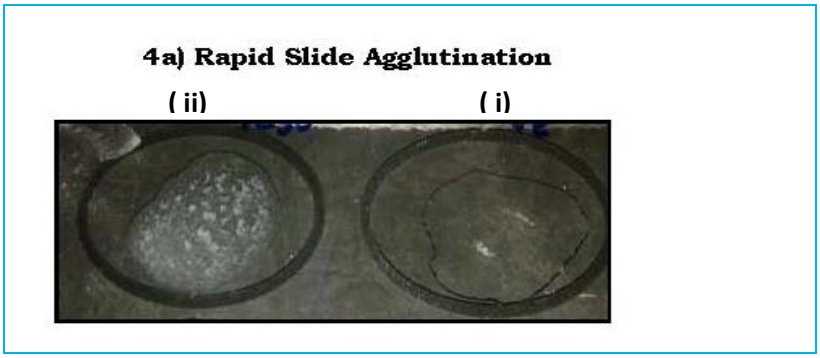

Fig: 4 a) (i) IgY from non-immunized chicken egg yolk was used as control which showed no agglutination with the whole cell antigen of *C.difficile*.
(ii) IgY from hyper –immunized chicken egg yolk showed visible agglutination with whole cell antigen of *C.difficile*. The binding of IgY was visualized by agglutination.

Determination of Specific Antibody Titre by ELISA: The specific antibody level in chicken serum and the titer of anti-*Clostridium difficile* – IgY in 1000-fold dilution of IgY-extracts obtained from the eggs of chicken immunized with *C. difficile* antigens were determined by Indirect ELISA as described by Lee *et al.*, (2002) using bacterial whole cells as antigen and mentioned as optical density (OD) at 405nm. The level of specific antibodies against the respective antigen in serum was increased after 1 week and slowly it reached the maximum titer on 56th day from the date of initial immunization (Fig 6).

However, the specific antibody level in the egg yolk was very weak on 21st day and gradually increased and reached the peak on 70th and 91st day. The titer of specific antibody was found to be 1:100000 on 91st Day and the titer were maintained with booster doses (Fig 4c). Results indicated that there was a delay in the appearance of anti- *Clostridium difficile* - IgY in yolk when compare to serum after the first immunization. It was possible due to the gradual accumulation of IgY during the yolk formation period by selective active transport (Kitaguchi *et al*., 2008).

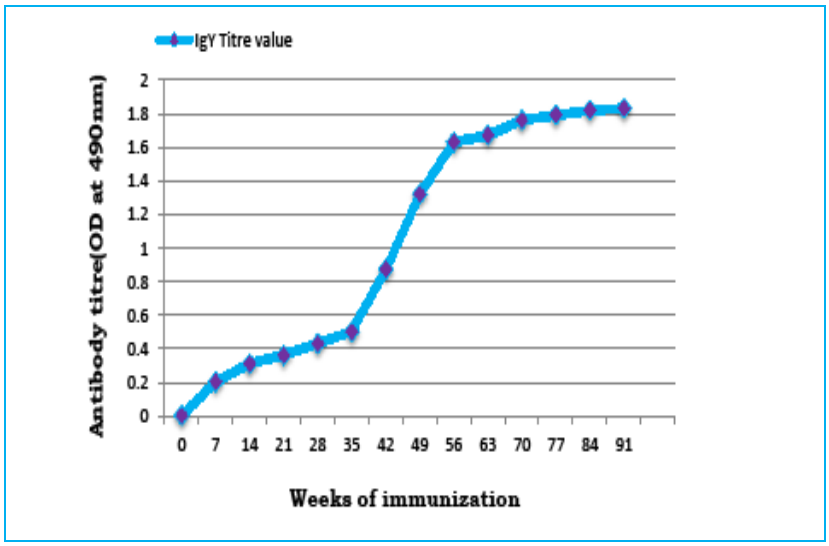

Figure 6: Dynamics of Antibody Production in Hens Immunized with *Clostridium difficile*

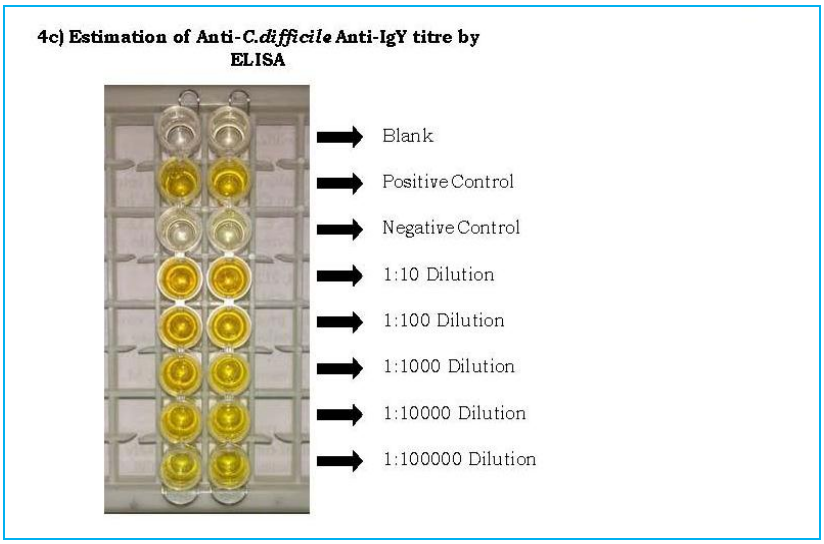

Fig: 4 c) The positive reaction shows up to 1: 100000 dilution indicates high amount of antibodies are produced in immunized chicken egg yolk

Protein profile by SDS – PAGE: The purified chicken egg yolk antibodies and its molecular weight were determined by Sodium Dodecyl Sulphate Polyacrylamide gel electrophoresis (SDS- PAGE) using 10% polyacrylamide gel at 100V and 10mA according to the method of Laemmli (1970). The SDS- PAGE shows a single band with a molecular weight of 180 KDa in each lane. A standard molecular protein marker was also run in parallel along with IgY fractions [Fig: 4b].

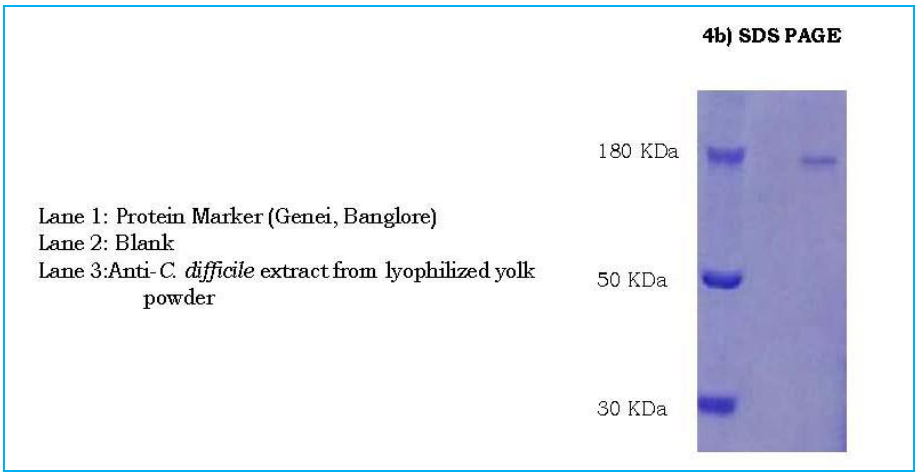

Fig: 4b) Coomassie brilliant blue- stained SDS-PAGE (10% gel) Protein profile antibody fractions. A single protein band of high molecular weight (180KDa) shows the purity of IgY in Lane 3

The electrophoretic band pattern obtained in this study revealed that the purity of IgY was higher in IgY fraction purified by Polson *et al.*, 1980 method. In order to evaluate the efficacy of IgY-*C.difficile* in preventing and treatment of *C. difficile* infection and the stability in humans was investigated at different physiochemical properties.

Physicochemical Properties & Stability of IgY

The assessment of stability of IgY was done by treating the IgY with various temperature, pH and pepsin treatment effect was assessed by measuring the residual activity using ELISA after each treatment. And this work was also done with IgY powder which is used to find the stability of IgY in the gastric environment of humans. The results showed that the purified IgY was stable at 4°C, 10°C, 25°C and 37°C. Approximately 25% of its activity was lost at 60°C and then significantly decreased at 70°C.(Fig:7)

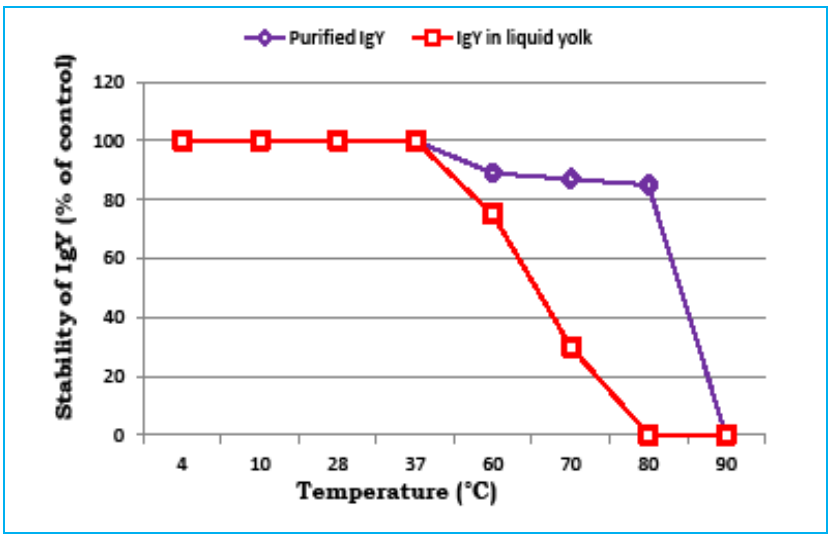

Figure 7: Thermal Stability of IgY

The purified IgY was stable between pH 4.0 and pH 8.0, at pH10 there was 70% activity and 20% activity at pH 2.0 and completely lost its activity at pH -12.0.(Fig:8)

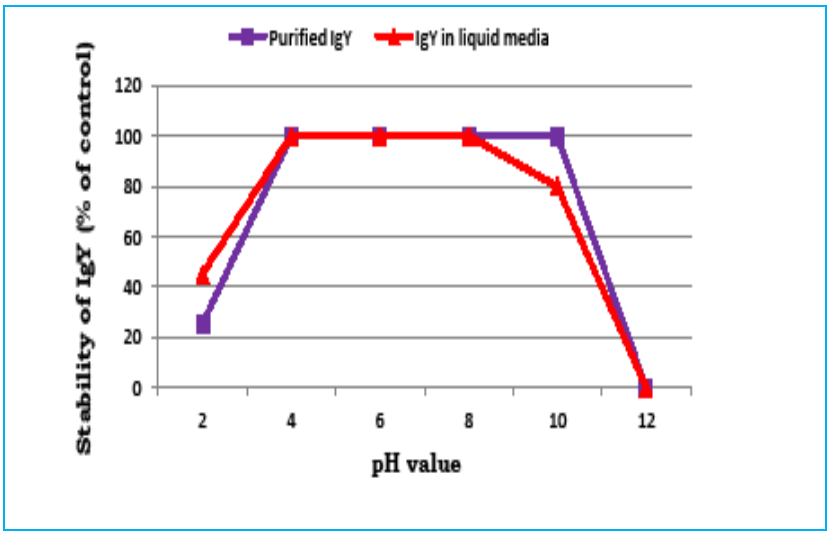

Figure 8: Stability of IgY at various pH

The results revealed that IgY was relatively stable to high temperature and broad pH range in the presence of its purified form and IgY was resistant to the effects of pepsin in the pH4.0.(Fig:9)

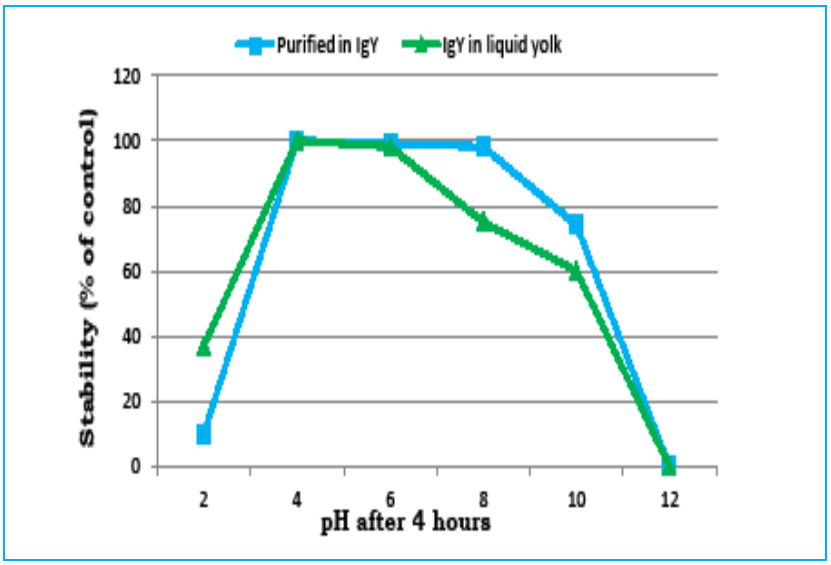

Figure 9: Stability of IgY of various pH on pepsin treatment

These observations indicate that once the IgY passes the acidity of the stomach, it could retain more than half of its activity and therefore, can minimize the effect of intestinal pathogens such as *Clostridium difficile*.

ii) In Vitro efficacy of *C.difficile* IgY by Growth inhibition Assay: In- vitro efficacy of anti-*C. difficile* IgY was determined by growth inhibition assay.

The objective of the assay was to determine whether the specific binding activity of anti-*C. difficile* IgY could inhibit the *C. difficile* growth in the liquid medium. The growth curve of *C.difficile* was plotted until the stationary phase reached at OD 600nm (Fig: 10)

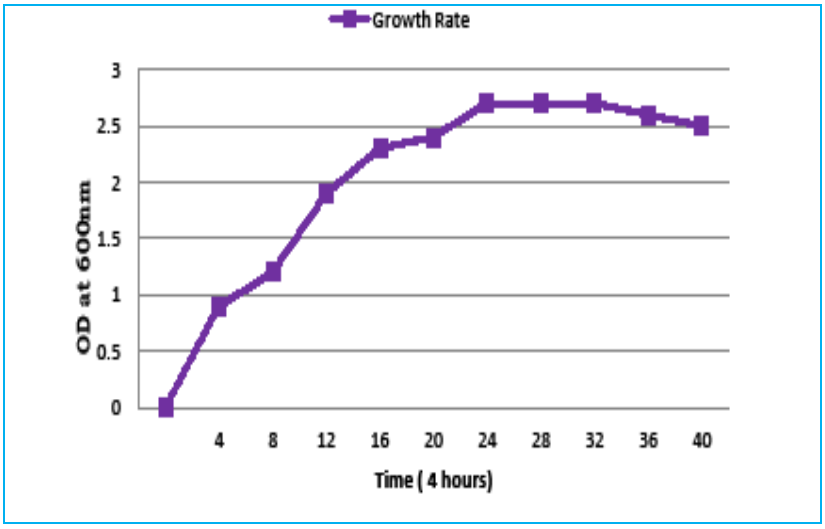

Figure 10: Growth curve of *C. difficile*

C.difficile was grown in BHI broth along with *C.difficile* –specific IgY and non-specific IgY were separately and incubated overnight at 37°C with shaking. Then samples were taken at 4 hours intervals to perform the growth inhibition assay by measuring the optical density. The growth of *C.difficile* incubated with specific IgY and non- specific IgY was correlated with that of the normal growth pattern of *C.difficile* in BHI broth. The growth of *C. difficile* with specific IgY showed significant reduction after 16 hours of incubation. (Fig: 11)

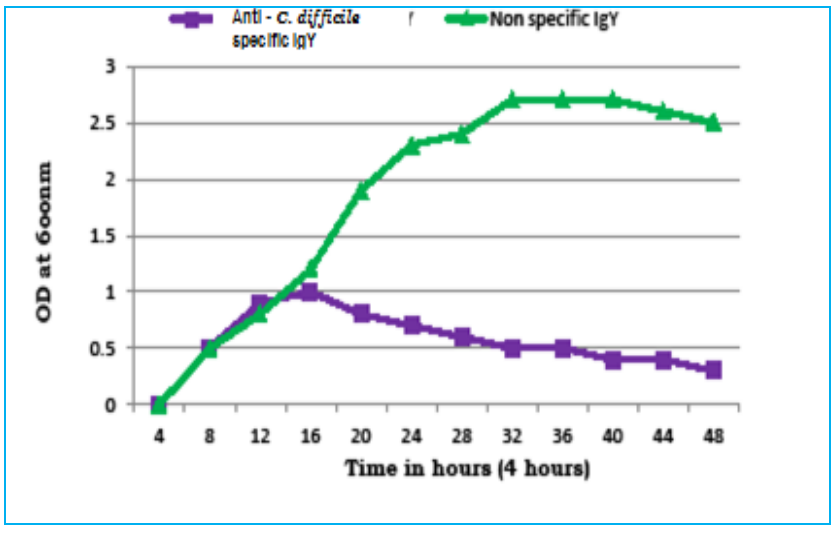

Figure 11: Growth Inhibitory Assay of *C. difficile*

It was observed that *C. difficile* -specific IgY were found to inhibit the growth of homologous cells in a liquid medium.

Antigen specific antibody has been widely used for immunological analysis in the field of diagnosis as well as pure scientific research, where the *C. difficile* can be harvested and used for preparation of IgY antibodies. IgY technology should be considered as a good alternative for treatment of infections. In the future studies the antibody that harvested against antigenic component of *C. difficile* can be incorporated as prophylactic and therapeutic agent. However more intense studies are to be done for successful application of egg derived antibody as a tool to control infection due to gastro intestinal disease in human.

CONCLUSION

Clostridium difficile infection (CDI) is now a major problem in hospitals worldwide. The bacterium causes nosocomial and antibiotic-associated disease which manifests itself in several forms ranging from mild self-limiting diarrhea to potentially life-threatening, severe pseudo membrane colitis. In the past ten years, variant toxin-producing strains of *C. difficile* have emerged, that have been associated with severe disease as well as outbreaks worldwide. Current therapies for the treatment of *C. difficile* infection rely on the use of antibiotics, notably metronidazole and vancomycin. However, these antibiotics are not effective in all cases and 20-30% of patients suffer relapse of the disease. Multiple alternate strategies are needed to tackle the increasing problem of drug resistance. Passive immunization against *Clostridium difficile* to prevent the colonization of gut mucosa and to clear the infection has been tried in small trials with reasonable success. IgY antibodies produced from the egg of chickens is an established, effective and easy source of antibodies. The ease of large scale production with encouraging efficacy makes the IgY antibodies an attractive treatment strategy to prevent and treat *Clostridium difficile* infection in humans. IgY technology, including the production and use of polyclonal IgY antibody (Ab), is a highly innovative and an expanding branch in human and veterinary medicine. Chicken eggs present an ideal alternative antibody source to mammals, as the IgY in the chickens' blood is transported to the egg and accumulates in the egg yolk in large quantities. Hens usually lay about 280 eggs in a year. Egg yolk contains a considerable amount of IgY, around 100-150 m/egg (Rose *et al*., 1974). Therefore, an immunized chicken yields more than 40 g of IgY a year through eggs, equivalent to that from 40 rabbits. In the sense of animal welfare, the use of laying hens for antibody production represents a refinement and a reduction in animal use. It is a

refinement in that the painful and invasive blood sampling or scarifying are replaced by collecting eggs. Hence, the present investigation is focused to generate chicken antibodies against *Clostridium difficile***.** In order to evaluate the efficacy of IgY- *Clostridium difficile* in preventing and treatment of *Clostridium difficile* infection and the stability in humans was investigated at different physiochemical properties.

Antibiotics are not only important to cure infections, but also to minimize the large risk from infections during surgery, transplantation or cancer chemotherapy. Although resistance is increasing, development of new antibiotics is also decreasing. Complements to antibiotics are urgently needed and passive immunization with IgY as the potential to be a complement. Passive immunization by oral administration of specific antibodies has been an attractive approach against gastrointestinal tract (GIT) pathogens in both humans and animals.

These studies indicate that eggs from chickens immunized with appropriate antigens are potentially a useful of passive immunity. Oral administration of IgY has proved successful for treatment of a variety of GI tract infections.

Antigen specific antibody has been widely used for immunological analysis in the field of diagnosis as well as pure scientific research, where the *Clostridium difficile* can be harvested and used for preparation of *IgY* antibodies. IgY technology should be considered as a good alternative for treatment of infections. In the future studies the antibody that harvested against antigenic component of *Clostridium difficile* can be incorporated as prophylactic and therapeutic agent. However more intense studies are to be done for successful application of egg derived antibody as a tool to control infection due to gastro intestinal disease in human. Finding an effective way to protect the antibodies from degradation in the GI tract would open the door for significant advances in IgY technology and nutraceutical applications. Passive immunotherapy with specific IgY may be a promising alternative with high specific nature and low cost effective.

CONFLIT OF INTEREST: The authors declare that they have no conflict of interests

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REFERENCE

1. Job, M.L & Jacobs, N.F; Jr Drug –induced Clostridium difficile associated disease. Drug Saf, 1997; 17: 37-46
2. Voth, D. &Ballad, J.D Clostridium difficile toxins: Mechanism of action and role in disease. Clin Microbiol Rev 2005; 18: 247-263.
3. Chung CH, Wu CJ, Lee HC, Yan JJ, Chang CM, *et al*., Clostridium difficile infection at a medical centre in southern Taiwan: Incidence ; Clinical features and prognosis. J. Microbiol Immunol Infect 2010; 43: 119-125.
4. LooVG, Bourgault AM, Poirier L, Lamothe F, Michaud S, *et al* Host and Pathogen factors for Clostridium difficile infection and colonization. N Engl J Med 2011; 365: 1693 – 1703
5. Kelly CP, Lamont JT Clostridium difficile more difficult than ever. N. Engl J Med 2008; 359: 1932 – 1940.
6. Hung YP, Tsai PJ, Hung KH, Liu HC, Lee CI, *et al*., Impact of toxigenic *C.difficile* colonization and infection among Hospitalized Adults at a District Hospital in Southern Taiwan. PLOS One 2011; e42415.
7. Kyne L, Hamel MB, Polavaram R, Kelly CP. Health Care Costs and mortality associated with nosocomial diarrhea due to *C.difficile*. Clin Infect Dis. 2002; 34:346-53
8. Barlett JG. Clinical Practice Antibiotic Associated Diarrhea. N. Eng J Med 2002; 346: 334-9.
9. Mojca Narat. Food Technol Biotechnol .2003; 41(3): 259- 267.
10. Han J, Hendrickson C. ILAR 2005; 46: 294-299.
11. Bizhanov, Jonauskiena I, Hau F. Scand J Lab Anim Sci 2004; 31: 121-130
12. Larson A, Balow RM, Lindahl TL, Forsberg PO. A review Poultry Scie. 1993; 72: 1807-1812.
13. Polson A, Von Wechmar MB and Van Regenmortel MHV Isolation of viral IgY antibodies from yolks of immunized hens. Immunol.Com, 1980; 9: 475 – 493.
14. Lowry O.H., Rosebrough N. J., Farr A. L., and Randall R. J., Protein measurement with the Folin phenol reagent. J. biol. Chem1951; 193: 265,
15. Laemmli U.K., Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 1970; 227 (5259): 680-685.