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Production of Oral IgY Antibody- A Novel Immunotherapy against *Clostridium Difficile*

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ABSTRACT

Clostridium difficile is the leading cause of nosocomial infectious Antibiotic – Associated Diarrhea (AAD). Like most other enteric bacterial pathogens, Clostridium difficile causes disease with a wide spectrum of severity ranging from mild 'nuisance' diarrhea with a normal colonic mucosa to pseudo membranous colitis, which is characterized by severe abdominal pain, diarrhoea and fever. Constitutional signs such as fever, fatigue and loss of appetite are prominent. The incidence of infection with this organism is increased in hospitals worldwide, due to the use of broad-spectrum antibiotics. Clostridium difficile is transmitted from person to person by the fecal-oral route. However, the organism forms large numbers of heat-resistant spores which remain viable in the hospital or nursing home environment for long periods of time. Infection rates for Clostridium difficile are reported to be around 10% after 2 weeks of hospitalization but may reach 50% after 4 or more weeks. Each year in North America, 1–3% of hospitalized patients receiving antibiotics become infected with C. difficile, leading to thousands of deaths and over $1 billion in associated costs to the health-care system.

KEYWORDS: *Clostridium difficile, IgY, SDS-PAGE, ELISA*

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INTRODUCTION

The term Antibiotic-Associated Diarrhea (AAD) refers to a benign, self-limited diarrhea following the use of antimicrobials. *Clostridium difficile* is the leading cause of nosocomial infectious Antibiotic – Associated Diarrhea (AAD). Like most other enteric bacterial pathogens, *Clostridium difficile* causes disease with a wide spectrum of severity ranging from mild 'nuisance' diarrhea with a normal colonic mucosa to pseudo membranous colitis, which is characterized by severe abdominal pain, diarrhea and fever. Constitutional signs such as fever, fatigue and loss of appetite are prominent. The occurrence of AAD varies greatly and is influenced by a number of factors, including nosocomial outbreaks, patterns of antimicrobial prescription and individual susceptibilities estimated that 10 -15% of all hospitalized patients treated with antibiotics will develop AAD. Extreme conditions of AAD leads to Pseudo membranous colitis (PMC), patients with PMC possess histological lesions. Most of these lesions revealed that the pseudo membrane is composed of fibrin, mucin, sloughed mucosal epithelial cells and acute inflammatory cells. The initial lesion has focal necrosis and inflammation as well as the characteristic 'summit'. The most advanced disease involves the complete structural necrosis with extensive involvement of lamina propria which is overlaid by a thick confluent pseudo membrane. Fecal micro biota transplantation (FMT), also commonly known as "fecal bacteriotherapy", represents the one therapeutic protocol that allows the fastest reconstitution of a normal composition of colon microbial communities. Surgery is a last resort for the treatment of unmanageable C. *difficile* associated diarrhea (CDAD) with toxic mega colon or colon perforations. The most frequent surgical techniques are either hemicolectomy or total colectomy. *C.difficile* is difficult to vaccinate since it operates the gut lining of humans and this zone is remote from the blood stream. Even if vaccination is effective it is too slow, taking 2 – 3 weeks to generate sufficient antibodies. So oral antibody composition can be ingested immediately by patient with rapid immune response. Hence IgY technology is a method used for production of polyclonal antibody in chicken. The egg provides a continuous source of monospecific polyclonal antibody and can be stored at 4ºC over ten years without any significant loss in antibody activity. The yolk of eggs laid by immunized chickens has been recognized as an excellent source of polyclonal antibody. This simple, non-invasive approach presents an appealing alternative to conventional polyclonal antibody methods. Purification of chicken IgY required no animal bleeding. A single chicken can produce enormous amount of antibody up to 3gm of IgY per month (10 – 20 times the amount of rabbits).

OBJECTIVE

- To prepare whole cell antigen of standard strain of *Clostridium difficile.*

- To immunize the 21-week White Leghorn chicken with the whole cell antigen of *Clostridium difficile.*
- To purify and characterize the Anti- *Clostridium difficile* chicken egg yolk antibody.
- To estimate the protein concentration of the chicken egg yolk antibody.
- To evaluate the specificity of the chicken egg yolk antibody by ELISA.
- To estimate the protein profile of Chicken IgY by SDS-PAGE.

REVIEW OF LITERURE

A. Antigenic structure

Toxins:

C.difficile can be divided into 24 toxin types based on the changes in both toxin genes. Some toxin types possess a third kind of toxin known as the binary toxin (Rupnik and Stubbs, 2000).

Toxin A:

The villus tips of the epithelium are initially disrupted followed by damage to the brush border membrane (Katyal *et al*., 2002) which is accompanied by extensive neutrophil infiltration resulting in massive inflammation. The fluid response is partly an outcome of the damage to the intestinal epithelium. Toxin A initially induces cell rounding which results in detachment of the cell from the basement membrane, followed by apoptosis. Toxin A also brings about a rapid loss of resident cells such as macrophages, T cells and eosinophils and induces changes in the shape of adherent polymorpho nuclear leukocytes. At least two pathophysiologic pathways are involved in changes in the epithelial cell barrier via glycosylation of the Rho proteins. These are

- Disaggregation of actin microfilaments leading to epithelial cell destruction and opening of tight junctions, and
- Early release of proinflammatory cytokines from intestinal epithelial cells probably via activation of mitogen-activated protein kinase.

The spherical cells become thin and rope like with rearrangement of F-actin cytoskeleton into aggregates (Britto *et al.,* 2002). Thus, the toxins alter the actin cytoskeleton, cause epithelial cell damage and result in increased permeability of the tight junctions. A severe acute necro-inflammatory reaction is produced by toxin A in the intestine resulting in activation of mast cells, vascular endothelium, and immune cells (Pothoulakis *et al.,* 2001).

Toxin B:

Both toxins disrupt the function of the Rho family of protein. Decreased transepithelial resistance and increased flux of paracellular marker such as mannitol and raffinose indicate the disruption of the tight junction (Hecht, 1992).

Binary toxin:

Another toxin, which is an iota-like toxin, was described and has been named binary toxin CDT. Binary toxin contains both toxins A and B and is a product of both toxin genes (*cdt B* for the binding component and *cdt A* for the enzymic component) (Popoff *et al., 1988*). It has not been found to be essential for eliciting *C. difficile* associated colitis. Binary toxin CDT is produced by most of *C. difficile* isolates with mutations in the *tcd A* and *tcd B* genes (Rupnik *et al*., 2002).

Surface proteins of *C. difficile*:

They demonstrated that SLPs play a role both in the initial colonization of the gut by *C. difficile* and in the subsequent inflammatory reaction. Different adhesins implicated in the colonization process of *C. difficile*are (*i*) flagella, composed of the flagellin Fli C and the flagellar cap protein Fli D, involved in cell and mucus attachment (*ii*) a cell-surface protein with adhesive properties, Cwp 66 (*iii*) a fibronectin-binding protein, Fbp68 and (*iv*) S-layer protein. These adhesins are able to induce an immune response, which could play a role in the defense mechanism of the host (Pechine *et al*., 2005). The cwp84, a surface protein exhibited proteolytic activity which could contribute to the degradation of the host tissue integrity and to dissemination of the infection (Janoir *et al*., 2007).

Flagella:

Flagella mediate chemotaxis of the vegetative cell and penetration of the mucus layer enabling adherence of the bacterium to the epithelial cell surface. There isless variation in flagella proteins (FliC& FliD) across*C. difficile* strains than other surface-associated proteins but they are highly immunogenic and are recognized by epithelial cells as part of the host cell pathogen-sensing pathway and can trigger downstream inflammatory responses (Collignon *et al*., 2005).

Pathogenesis:

Patients with a leukemoidreaction have a mortality rate of approximately 50%, significantly higher than that of other forms of CDAD (Bouza *et al.,* 2005).*C. difficile*, like virtually all bacterial enteric pathogens, causes a spectrum of clinical conditions with both colonic and extracolonic manifestations. The different manifestations are detailed below.

Asymptomatic carriage:

Colonization with *C. difficile* is the presence of the organism in a person with no clinical symptoms like diarrhea. Asymptomatic carriage of *C. difficile*is quite common in hospitalized patients. Epidemiologic studies have reported that 10-16 per cent of hospital inpatients in high-risk units become carriers after receiving antibiotics (Johnson *et al*., 2001) suggested that asymptomatic carriers of epidemic and non-epidemic*C. difficile* isolates have the potential to contribute significantly to disease transmission in long-term care facilities. Asymptomatic carriage can be predicted by considering certain clinical factors such as recent antibiotic exposure or previous occurrence of CDAD.

***difficile* diarrhea:**

Usually mild to moderate diarrhea, sometimes accompanied by lower abdominal cramps is seen with *C. difficile*infection. Symptoms usually begin during or shortly after antibiotic therapy. Occasionally these may be delayed for several weeks. *C.difficile* toxins can be usually detected from faecal specimens, even though endoscopic and histologic features may be normal in patients with mild disease. The diarrhoea resolves with the stoppage of antibiotics (Farrell *et al.,* 2000).

***difficile* colitis:**

Sometimes dehydration and a low-grade fever with a systemic polymorphonuclear leukocytosis may occur. Levels of lactoferrin released from the secondary granules of intestinal leukocytes, as well as other inflammatory markers rise significantly in patients having advanced CDAD compared to patients with a milder form of the disease. Faecal lactoferrin assay performed simultaneously with the *C. difficile*toxin assay can help rule out asymptomatic carriage of *C. difficile*. A nonspecific diffuse or patchy

erythematous colitis without pseudomembrane may be seen under sigmoidoscopy (Vaishnavi *et al.,* 2000).

Pseudomembranous colitis (PMC):
The classic pseudomembranes, which are raised yellow plaques ranging from 2-10 mm in diameter scattered over the colorectal mucosa are best revealed by sigmoidoscopic examination. White blood cell counts of 20,000 or greater and hypoalbuminemia of 3.0 g/dl or lower may be observed in severely ill patients (Jebhard *et al*., 1983).

Fulminant colitis:
Further morbidity and mortality can be prevented in patients with fulminant *C. difficile* colitis by aggressive diagnostic and therapeutic interventions. Though the risks of perforation are generally uncommon, limited flexible sigmoidoscopy or colonoscopy may be performed at the bedside (Vaishnavi *et al*., 2003). *C. difficile* infection has also been reported to be involved in the exacerbation of ulcerative colitis.

Recurrent CDAD:
Recurrent CDAD is a difficult clinical problem due to repeated recurrences of the manifestation. The pathophysiology is not quite clear and may be due to persistently altered faecal flora. Repeat antibiotics may subsequently be unable to suppress *C. difficile* overgrowth. Alternatively, impaired immune response may also be responsible. It has been estimated that approximately 15-20 percent of patients treated for CDAD, relapse following successful therapy. This condition is manifested by the sudden re-appearance of diarrhea and other symptoms usually within a week of stopping treatment with vancomycin or metronidazole. Patients who relapse once are at greater risk of further relapses. Relapse is generally not related to antibiotic resistance because in some patients re-infection can occur with the same or different strain (Kelly *et al*., 1994).The small bowel and the appendix may also act as reservoirs of *C.difficile* spores that enter the colon and result in relapse.

Extracolonic features:
Recent literature mentions that CDAD is no longer limited to the colon. *C. difficile* may infrequently cause disease in a variety of other organ systems and except for bowel involvement and reactive arthritis most of the cases do not appear to be strongly related to previous antibiotic exposure though they are preceded by specific or nonspecific gastrointestinal (GI) disease. Some of the features of extracolonic diseases can be summed up as follows:

Small bowel:
The small intestinal *C. difficile* infections seem to be increasing in incidence (Jacobs *et al.,* 2001*).* Small bowel CDAD with formation of pseudo membranes on ileal mucosa may occur when previous surgery on it has been carried out and is associated with a high mortality rate (Testore *et al*., 1986) examined jejunal specimens from 100 patients who died without any immediate history of GI symptoms and mucosal cultures in 3 cases treated with antibiotics were positive for *C. difficile*.

Bacteremia:
Like with other colonic bacteria, *C. difficile* is also known to cause bacteremia with about 20 per cent mortality and also reported a unique case of monomicrobial *C. difficile*bacteremia in a young woman with an underlying hematologic malignancy but without any GI symptoms (Libby, 2009).

Reactive arthritis:
C. difficile related polyarticular kind of reactive arthritis may involve joints of the knee and wrist in about a 50 per cent of the cases. Reactive arthritis begins an average of 11.3 days after the onset of diarrhoea and is a prolonged illness, taking an average of 68 days to resolve (Birnbaum *et al.,* 2008).

Miscellaneous entities:
Other extracolonic manifestations due to *C. difficile* include cellulitis, necrotizing fasciitis, osteomyelitis, prosthetic device infections, intra-abdominal abscess, empyema, localized skin infections, *etc*.

B. Factors precipitating CDAD:
The following factors determine whether or not a patient develops a *C. difficile* infection:

General factors:
Patients are at continuous risk of exposure to *C. difficile* during the period of hospitalization and become vulnerable to infection after they have been exposed to antimicrobials. The two most important components essential for CDAD is exposure to antimicrobials followed by exposure to *C. difficile* and majority of the patients do not get ill with these till the third additional factor related to host immunity, virulence of infecting *C. difficile* strain or to type and timing of exposure come into play.

Specific factors:
Thus, the combination of the environmental presence of *C. difficile* in health care settings and the number of people receiving antibiotics, immunosuppressive, PPI or cancer therapeutics in these settings can result in frequent outbreaks (Gellad *et al.,* 2007).

Diagnosis:
The proper laboratory specimen for the diagnosis of *C. difficile* infection is a watery, loose, or unformed stool. Because 10% or more of hospitalized patients may be colonized with *C. difficile,* evaluating a formed stool for the presence of the organism or its toxins can decrease the specificity of the diagnosis of CDI. Processing a single specimen from a patient at the onset of a symptomatic episode usually is sufficient. Because of the low increase in yield and the possibility of false-positive results, routine testing of multiple stool specimens is not supported as a cost-effective diagnostic practice.

Detection by cell cytotoxicity assay:
Detection of neutralizable toxin activity in stools from patients with antibiotic-associated colitis was the initial observation that led to the discovery that *C. difficile* is the causative agent of this infection. The presence or absence of the pathogenicity locus (PaLoc), a 19-kilobase area of the *C.difficile* genome that includes the genes for toxins A and B and surrounding regulatory genes, accounts for the fact that most strains of *C. difficile* produce either both toxins or neither toxin, although an increasing number of strains are found to lack production of toxin A. Numerous cell lines are satisfactory for detection of cytotoxin, but most laboratories use human foreskin fibroblast cells, on the basis of the fact that it is the most sensitive cell line for detecting toxin at low titer (1:160 or less).

Detection by EIA for toxin A or toxins A and B:
Commercial EIA tests have been introduced that either detect toxin A only or detect both toxins A and B. Compared with diagnostic criteria that included a clinical definition of diarrhea and laboratory testing that included cytotoxin and culture, the sensitivity of these tests is 63%–94%, with a specificity of 75%–100%. These tests have been adopted by more than 90% of laboratories in the United States because of their ease of use and lower labor costs, compared with the cell cytotoxin assay. The toxin A/B assay is preferred because 1%–2% of strains in the United States are negative for toxin A (Geric, 2003).

Detection by culture:
Along with cytotoxin detection, culture has been a mainstay in the laboratory diagnosis of CDI and is essential for the epidemiologic study of isolates. The description of a medium containing cycloserine, cefoxitin, and fructose (CCFA medium) provided laboratories with a selective culture system for recovery of *C. difficile*. Addition of taurocholate or lysozyme can enhance recovery of *C. difficile,* presumably because of increased germination of spores. Optimal results require that culture plates be reduced in an anaerobic environment prior to use. The strains produce flat, yellow, ground glass–appearing colonies with a surrounding yellow halo in the medium. The colonies have a typical odour and fluoresce with a Wood's lamp. Additionally, Gram stain of these colonies must show typical morphology (gram positive or gram variable bacilli) for *C. difficile* (Wilkins, 2003).

C. Other test methodologies:
Pseudo membranous colitis can only be diagnosed by direct visualization of pseudo membranes on lower gastrointestinal endoscopy (either sigmoidoscopy or colonoscopy) or by histopathologic examination. However, direct visualization using any of these techniques will detect pseudo membranes in only 51–55% of CDI cases that are diagnosed by combined clinical and laboratory criteria that include both a culture positive for *C. difficile* and a positive stool cytotoxin test result. Pseudo membranous colitis has been used as a marker of severe disease, as has CT scanning. Abdominal CT scanning may facilitate the diagnosis of CDI but this methodology is neither sensitive nor specific (Farrell, 2000).

D. Treatment:
Biotherapy (therapy involving probiotics) is emerging as a potential means of controlling *C. difficile* diarrhoea recurrences. The role of the probiotic organisms is to restore the colonization resistance of the normal flora, disrupted by the effects of antibiotic therapy, in order to prevent re-infection by *C. difficile.* The most promising probiotic agent is *Saccharomyces boulardii*, a live nonpathogenic yeast shown to have some benefit in the treatment of AAD by binding to the glycoprotein receptor site for toxin A at the intestinal brush border (Persky *et al*., 2000). *LactobacillusGG*, another popular probiotic, has been shown to improve intestinal immunity by increasing IgG and IgA levels at the intestinal mucosal level and may be effective against CDI (Pochapin, 2000) Surgical treatment of *C. difficile* colitis is reserved for patients with complicated CDI, toxic megacolon and those who do not respond to medical therapy. Surgical treatment is associated with significant morbidity and carries a mortality rate close to 40%. Comorbidity and late intervention are largely responsible for the poor outcomes of these patients.

E. Immunoglobulins:
During the past 20 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 (Hau *et al.,* 2005).

Stability:
The half life time of Ig Y is in months and that they retain their activity after 6 months at room temperature or for one month at 37º C. Experience with yolk antibodies is that they are stable overtime, which contradicts some rumors that chicken antibodies are labile Ig Y antibody have been stored for over 10 years at 4º C without any significant loss in antibody activity (Shimizu *et al.,* 1992).

Phases of immune response:
The antigen- specific immune response may be divided into three phases termed as inductive phase, effector phase and the establishment of immunological memory. In the inductive phase, an antigen is recognized as foreign by so called antigen presenting cells. The next step in the immune response is the effector phase. Depending on the type of antigen, activation of T cells leads to cell mediated responses (mainly cytotoxic). The final phase of the antigen specific immune response is the induction of memory after primary contact with the antigen. Memory which is based on the differentiation of B cells, gives the secondary immune response its characteristics of more speed and greater magnitude than the initial response.

F. Immunization of chickens:
Immunization schemes that have been developed for other species (e.g., rabbits) normally apply well in the chicken and most producers of IgY use adjuvant. Adjuvants are used to stimulate the immune response of experimental animals; the desired antigen is applied in combination with various adjuvant compounds. The ideal adjuvant can be characterized as a substance which stimulates high and sustainable antibody titers even with small quantities of antigen (Hodek *et al*., 2003). Water-in-oil emulsions, which include Freund-type adjuvants, are the adjuvants most commonly used to produce antibodies in laboratory animals.

Transfer of IgY from hen to egg:
Moreover, FcRY-expressing cells transcytosed the receptor at pH 6, but not at pH 8. The egg yolk is slightly acidic (pH 6) which should favor binding of the FcRY to IgY, and when the complex reaches the blood with a more basic pH (pH 7.4) IgY is released. An intact Fc region is required for uptake into the egg yolk, and IgY has several amino acid sequences that are also of importance. IgY concentration during maturation of the egg is steady, and the amount of immunospecific IgY corresponds linearly to the hen's serum concentration (Bollen *et al.,* 1997). All subpopulations of maternal IgY are transferred into the chicken oocyte (Loeken *et al.,* 1983).

Antibody production:
After an initial immunization, booster doses are often given to assure high antibody titers over time. Typically, after the first immunization one or two booster doses are given every fourth week and thereafter every other month. It takes about 6–7 days after detection in serum until specific antibodies are found in the egg yolk. A small amount of antigen elicits a strong immune response, but the response depends on the

type of antigen and the hen. IgY antibodies have high avidity and the antibody titers are persistent over time (Lemamy *et al.*, 1999). One laying hen produces approximately 20 eggs per month, and each egg contains a high concentration of IgY, about 100 mg. Thus, over 2000 mg IgY per month can be isolated whereof up to 10% is specific for the antigen used for immunization.

Purification:
The plasma fraction can be further divided into low-density lipoproteins and a water-soluble part. The water-soluble fraction contains live tins, among them IgY. There are several methods to purify IgY from egg yolk, e.g. salt, dextran sulphate, xanthan, gum, ethanol or polyethylene glycol (PEG) precipitation, thiophilic chromatography or water dilution(Akita *et al.*, 1992). Depending on the type of application and demand, more than one method can be combined.

G. Applications of avian vs. mammalian antibodies:
It is possible to obtain 5–10 times more antibodies from a hen than from a rabbit under the same period of time, making the production of avian antibodies cheaper than the mammalian equivalent. IgY antibodies are preferable since the number of animals is reduced and there is no need to bleed them. It is more practical to collect eggs than blood and much less harmful to the animals. Moreover, it is more practical to purify IgY from the egg yolk, since it does not contain any other immunoglobulins than IgY, compared to purification of IgG from sera, which contains other immunoglobulin classes as well as many other constituents. IgY purification is mostly about removal of egg yolk lipids. There are also other properties that make IgY highly interesting for oral immunotherapy.

IgY in immunological assays and diagnostics:
Due to the evolutionary distance between mammalian and avian antibodies, IgY is very well suited for immunological assays of mammalian proteins, especially since the risk for unspecific cross-reactivity and background can be minimized. The comparison of IgY to IgG has shown that IgY is as good as or even better than IgG in many immunoassays (Zhang *et al.*, 2003). IgY can be labeled, like IgG, with for example biotin with good activity and stability. IgY can also be used for diagnostic purposes. An example is anti-cystatin C IgY for detection of cystatin C, a marker of kidney function. IgY against recombinant rabies proteins has also been suggested as suitable for diagnosis.

IgY for oral immunotherapy:
This is in contrast to antigen-antibody complexes with mammalian IgG antibodies, which will activate the human complement system and produce potent inflammatory mediators. Orally administered immunoglobulins do not pass from the intestines to the blood, and thus there will be no systemic effects. In contrast, IgY given intravenously to mice elicited an anti-IgY response. IgY is well tolerated for oral immunotherapy, but less so for intravenous injections. There is essentially no risk that bacteria or other pathogens should develop resistance against oral treatment with IgY. It is comparable with eating raw egg yolk and IgY is an ingredient in our regular diet. Thus, it is completely safe as long as the subject is not allergic to eggs. Allergic reactions may occur to egg-derived antibodies and residual egg proteins (Bernhisel *et al.*, 1991).

Studies of IgY for immunotherapy:
Animal studies:
Moreover, IgY given intravenously as antivenom reduced the effect of snake venom, and the mortality in mice was reduced as well as edema. Animal hyperimmune sera are commonly used, but there is a risk of anaphylactic reactions and serum sickness. These reactions were not seen with the IgY antivenom (Meenatchisundaram *et al.,* 2008).

Human studies:
In one study the antibodies were given as a supplement in drinking yoghurt, which illustrates the possibility of using IgY in functional food. In a double-blind placebo-controlled study, (Burrows *et al.*, 2007)

H. Antimicrobial effects of IgY:
IgY binding to *Salmonella* alters the surface of the bacteria, which could explain the reduced ability to adhere (Sunwoo *et al*, 2002).

Antibiotic resistance and other side effect:
The spread of these bacteria is not only caused by antibiotic usage, but also by poor hygiene, primarily at care settings. Thus, infectious control is also important in minimizing the spread of antibiotic resistance. Other problems with antibiotics, besides resistance, are disturbance of the normal flora, which predisposes for infections by other opportunistic and pathogenic bacteria and fungi, and the risk for allergenic and toxic side effects of antibiotics. Vaginal candidiasis is associated with antibiotic use. Treatments with amino glycoside antibiotics have been linked to both nephrotoxicity and hearing loss. Antibiotics are not only important to cure infections, but also to minimize the otherwise large risk for infections during surgery, transplantation or cancer chemotherapy. Although resistance is increasing, development of new antibiotics is decreasing. (Hogberg *et al.*, 2008) Complements to antibiotics are urgently needed and passive immunization with IgY has the potential to be such a complement.

MATERIALS AND METHODS:
A. Experimental Animal:
White leghorn chicken:
Twenty-one weeks old White Leghorn chickens in good health condition were obtained from L.K poultry, Palladam. The chickens were maintained in our animal facility with normal feeding and used for the study.

Bacterial strain:
Bacterial strain used for the present study is *Clostridium difficile.* The standard strain was obtained from ATCC(NO: 9689).The strain was cultured an aerobically on Blood agar plates with repeated sub culturing and stored for further analysis.

Sterilization:
In the experiments, standard techniques were followed for sterilization of media, glass wares, etc.

B. Characterization of *Clostridium difficile*:
Colony morphological observation:
The *Clostridium difficile* culture was inoculated into Nutrient broth and Brain Heart infusion broth. The broth culture was streaked by quadrant streak method on Blood agar. The plates were incubated an aerobically at 37^0C for 48 hrs. The colonies were observed for their appearance, elevation and pigmentation to characterize the isolates.

Microscopic observation:

Gram staining technique:

The grams reaction of the cells and the morphology of the cells were noted down.

Biochemical characterization:

Characterization of *C. difficile* strain:

The biochemical tests were performed following the Bergey's Manual of Determinative Bacteriology, to confirm the standard strain *Clostridium difficile*. The selected organisms were characterized biochemically using the following reactions. This helps to identify the isolated organism into respective genus and species. The *Clostridium difficile* were identified by the following tests.

Carbohydrate Fermentation test:

A sugar free Andrade peptone medium was prepared and dispensed in the test tubes and Durham's tube was introduced inverted into each test tubes. The medium containing peptone broth, Andrade indicator was sterilized at 121^{0}C for 15 lbs./inch square into pressure. Various sugars such as arabinose, glucose, fructose, lactose, maltose, mannitol, starch, sucrose and sorbitol at a concentration of 1% were filter sterilized and added to the sterile medium. The test organisms were inoculated in the test tubes and incubated at 37^{0}C for 24 hours.

Catalase Test:

A loopful of culture was transferred to the surface of a clean, dry glass slide. Immediately place 1 drop of 3% hydrogen peroxide on to the organism on the slide. The slides were observed immediately for the formation of bubbles indicating oxygen production which is positive. No bubble formation indicates the negative result.

Toxicity Testing:

In a test tube, 200µl of sample diluent was added using the dropper assembly. 3 drops of Enzyme conjugate was added vertically to the test tube. 25 µl of the broth culture was added to the tube, vortexed and the tube was allowed to stand at 24-26º C for 5 minutes. 150 µl of the diluent sample was added to the immunocard ports (lower) and incubated at same temperature for 5 minutes. The wash buffer was holed vertically and added 3 drops on the upper port of the immunocard. Once wash buffer was absorbed, 3 drops of the substrate was added to both the reaction ports and incubated at same conditions for 5 minutes. The results were visually read after incubation.

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, 7.4). After the last wash, pellets were resuspended in PBS containing 1% (vol/vol) formaldehyde and incubated overnight. Excess formaldehyde was removed 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 resuspending an aliquot of the cell pellet in PBS saline and plating 199µl 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. The adjuvant used was Freund's complete adjuvant for primary immunization and Freund's in-complete adjuvant for booster immunization.

C. Immunization of 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 3×10^8 cells.

For first immunization, the 21 days old white leghorn chickens were intramuscularly injected at multiple sites of the breast muscles with antigen emulsified in FCA. 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. Further, eggs laid by the chicken under the test were collected regularly and stored at 4ºC.

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**		

D. Collection of eggs:

The eggs were collected daily, then cleaned and labeled with an identification number date using a waterproof pen. The collected eggs were refrigerated at 4^{0}C until the isolation of immunoglobulin. Yolk antibodies are stable *in situ* in the refrigerator for at least 6 months.

E. Purification of IgY Antibodies from Egg Yolk:

Separation of Egg-Yolk:

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 stick. 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).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^{0}C. 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.

F. Purification of Antibodies by Dialysis:

Bag activation for Dialysis

The dialysis bag was cut into pieces of required and convenient length and allowed to boil for 10 minutes in distilled water. The bags were transferred in to boiling 100 ml distilled water containing 2g of sodium bicarbonate and 0.0375g EDTA and boiled for 10 minutes. Then the bag was washed in boiling distilled water and transferred into 100ml distilled water containing 0.0375g EDTA and boiled for 10 minutes. Then the bags were rinsed thoroughly in distilled water using washing bottles and stored at 4^{0}C in 50% ethanol. Care was taken that dialysis bag was thoroughly submerged. After these steps, the dialysis bag was handled with gloves. Before use, the dialysis bag was washed inside and outside with distilled water.

Purification of IgY antibodies by Dialysis:

The egg yolk antibodies were desalted by dialysis against buffer "S" (without NaCl) to remove ammonium sulphate. The cellulose membrane tubing (pore size – 0.25µm) was cut into piece of required and convenient length. It was allowed to boil for 10 min in 2% (w/v) sodium bicarbonate and 1mM of EDTA (pH 8.0). After activating the tubing, it was handled with gloves. The tubing was cooled and rinsed thoroughly with water. Before use, the bag was opened and washed inside and outside with distilled water. A quantity of 5ml of IgY fractions were transferred to the dialysis membrane bag. The bag was twisted and tied at both the ends after inflated with some air. Then the bag was carefully immersed in a beaker containing buffer "S" (without NaCl) and the apparatus was maintained at 5^{0}C in a refrigerator. At every 12 hours of interval the buffer was replaced with fresh buffer. Buffers were frequently changed until the Ammonium sulphate gets eluted completely. The elution was confirmed with Nessler's reagent which forms brown precipitate in the presence of Ammonium sulphate. Finally, the contents were removed from the dialysis membrane bag after complete removal of Ammonium sulphate and Lyophilized. The IgY powder was stored under -20^{o}C and used for further studies.

Dialysis of IgY extract:

The IgY extract obtained from egg yolk was transferred to an active dialysis bag and tied after including some air and twisting the open end of the dialysis bag. The bags containing IgY extract was dialyzedovernight in 0.1% saline (1,600ml) and gently stirred by means of magnetic stirrer. Next morning, the saline is replaced by PBS and dialyzed for another three hours. Thereafter the IgY-extract was pulled from the dialysis capsule by a pipette and transferred to storage vials.

G. 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). A quantity of 10mg BSA was dissolved in 10ml of distilled water and used as stock solution. To a series of clean test tubes 0.2 – 1.0 ml of BSA (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-Ciocalteu reagent was added to each tube and incubated under dark condition for 30 minutes at room temperature. The OD was read at 660nm against the reagent blank and was plotted on a graph. Thus, the standard graph was obtained. Similarly, the OD values of IgY were also taken and compared with standard graph and tabulated.

H. 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.

I. 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). 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 detaining it with 7% acetic acid. This removes the dye that was not bound to proteins. The detainer was changed frequently until the background of the gel became colorless.

J. 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 reddishperoxidase 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.

RESULT:

A. Maintenance of Culture

The standard strain *Clostridium difficile* obtained from ATCC (NO.9689) was revived, sub-cultured and maintained in laboratory condition as per the standard protocols. Then the *C. difficile* culture was characterized by morphology, cultural characteristics on various culture media and biochemical characteristics.

Characterization of Standard Strains

Microscopic Studies

Gram's Reaction:

Thin smears were prepared on clean grease free glass slides. Smears were subjected to Gram's staining technique. Then the gram stained slides were observed under oil immersion objective in a light microscope. Gram positive, *Clostridium difficile* rods, with a long irregularly (drumstick/ spindle shaped) cells with a bulge at terminal ends were observed.

Spore staining:

Smear of the bacterial culture was made on clear glass slide. The smear was air dried and heat fixed. The smear was subjected to spore staining and observed under oil immersion objective lens. The spores were seen inside a pink colored sporangium.

Cultural characteristics:

The standard culture was grown in Blood agar. The colony character observed were tiny, translucent, gray colonies surrounded by a narrow zone of "soft" beta hemolysis. There was no growth on the MacConkey plate.

B. Biochemical Tests:

Carbohydrate fermentation test:

Carbohydrate Fermentation test was performed and the results were recorded.

Catalase test:

When 3% hydrogen peroxide was added to the culture no oxygen was produced in the form of bubbles. This shows that the organism is not capable of producing catalase.

C. Toxicity testing:

After the incubation time following the addition of enzyme substrate and the culture, the immunocard was observed for the results. The color formation on the ports of the card indicates the culture is positive for toxin production.

Generation of Antibodies in white leghorn Hens:

21-week-old white leghorn Hens were immunized intramuscularly with 0.5ml of *Clostridium difficile* whole cell antigen (containing 3 × 10^8 cells/ml). Booster doses were given in two week's intervals. The pre-immune sera and hyper immune sera were collected at specified time intervals during and after the various immunization schedules. The presence of antibody in chicken serum was assessed by slide agglutination method. After immunization the eggs were collected and stored at 4ºC with proper marking of name and date. 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 PEG method described by Polson *et al.,* 1980. Then the antibodies were subjected to dialysis, and stored for further studies.

Estimation of Protein concentration:

The protein content of the IgY was estimated by the Lowry *et al.,* (1951) using Folin-Ciocalteu reagent. The optical density of the BSA standard was used to plot the graph. Increased amount of protein content was observed in anti-*Clostridium difficile* antibodies from the chicken immunized with *C. difficile* whole cell antigens. The protein content was increased up to 21.15 mg/ml at 84thday and its total IgY concentration reached up to 6.98 mg/ml.

Table-2: Protein Estimation by Lowry *et al.,* (1951)

Daysof Egg Collection	Total Protein Concentration mg/ml	TotalIgY Concentration mg/ml
0	14.32	0.032
7	15.14	0.86
14	16.65	1.14
21	17.32	1.56
28	17.43	2.15
35	17.65	2.65
42	18.23	3.67
49	19.34	4.15
56	20.67	5.29
63	22.98	5.98
70	24.31	6.12
77	28.34	6.56
84	29.15	6.98

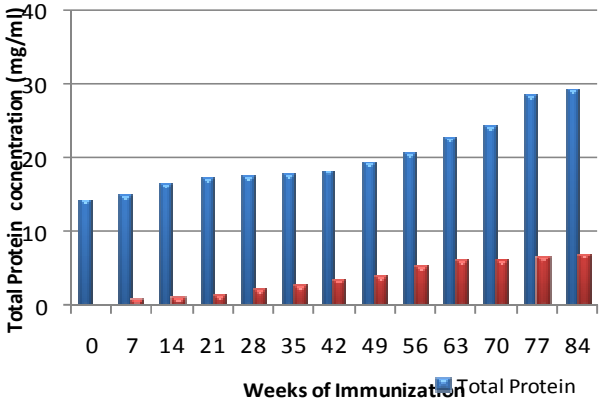

Figure-1: Total Protein & total IgY Concentration of egg-yolk of immunized chicken

D. Determination of Specific Antibodies in yolk and Serum:

The specificity of Anti-*Clostridium difficile* antibodies in the serum and egg yolk from immunized laying hens was determined by Rapid slide agglutination Test (RSA). Appearance of agglutination within 2minutes, when the antigen was mixed with the corresponding IgY, 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 with this qualitative determination further titration of the specific IgY could be performed by ELISA.

E. Determination of Specific Antibody Titre by ELISA:
The antibody titre potency of each IgY fractions obtained above was determined by the following modified ELISA as described by Lee *et al.,* (2002). The antibody titre increases at the time of booster injections, even a minute increase in antibody titre can be traced by this assay. The comparative results show that the antibody titre potencies changes in the courses of immunization. As it has been found that the antibodies against bacterial antigens moves efficiently from serum to an egg yolk and concentrated in the egg yolk. The rate of dilution of antibodies given an OD_{490} value in 1/10000 dilution.

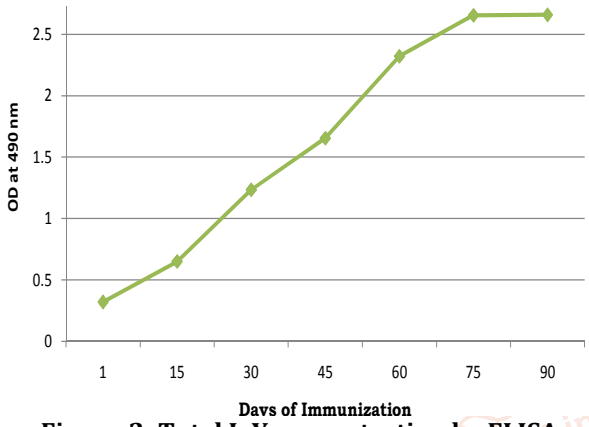

Figure-2: Total IgY concentration by ELISA

F. 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 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.

DISCUSSION:
C.difficile infection (CDI) is the leading cause of antibiotic-associated diarrhoea and a highly problematic healthcare-associated infection (HAI). Alteration of the normal lower intestinal microbiota by exposure to antibiotics provides an environment that allows *C. difficile* to multiply, flourish, and produce toxins that cause colitis (Lyres *et al*., 2009). Maintaining proper hand hygiene is considered to be the best method of prevention of AAD. Oral vancomycin and metronidazole used for 7-10 days are considered the first line of therapy by most clinicians and current guidelines (Burdon, 1979). Biotherapy (therapy involving probiotics) is emerging as a potential means of controlling *C. difficile* diarrhoea recurrences. The role of the probiotic organisms is to restore the colonization resistance of the normal flora, disrupted by the effects of antibiotic therapy, in order to prevent re-infection by *C. difficile.* But relapsing conditions are more common when the intake of the drug is stopped, and shows adverse effects. To overcome this problem IgY antibodies can be used.

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 chicken's 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 hen 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. The almost extreme properties of antibodies to recognize small specific structures on other molecules have made them a very useful tool in studying other molecules as well as complex reactions.

During the past 20 years, the use of chickens instead of mammals for antibody production has increased. There are many advantages of IgY compared to mammalian antibodies. For example, there is a great phylogenetic distance between birds and mammals, and hence, IgY has affinity to more epitopes of mammalian proteins than a corresponding mammalian antibody (Horton *et al.*, 1985). It is possible to obtain 5–10 times more antibodies from a hen than from a rabbit under the same period of time.

Previous studies have focused on the protection against *Clostridium difficile* Associated Diarrhoea (CDAD), by using the anti-toxin antibodies, anti-surface layer protein antibodies etc. The present study is focused to develop egg yolk antibodies against the whole cell antigen of *C. difficile*, which prevents the colonization *C. difficile.* 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 titre in the egg yolk. The eggs were collected, stored and antibodies were purified from chicken egg yolk by Polson *et al*., (1980) method. It was monitored that the increase in the specific antibody concentration of egg yolks from immunized hens over the course of immunization period. The antibodies against *C. difficile* was first appeared in serum on 7th day after starting the immunization schedule. Then the antibodies were detected in egg yolk after a week. The molecular weight of the purified IgYs were confirmed as 180KDa through SDS PAGE (Laemmli, 1970). The total protein concentration was estimated by the method described by Lowry *et al.,* (1951) using Folin-Ciocalteu reagent. The optical density of the BSA standard was used to plot the graph and the total protein concentration initially at 7th was found to be 15.14 mg /ml and later it was increased up to 29.15 mg/ml at 84th day. The total IgY concentration at 7th day was 0.86 mg/ml and it raised up to 6.98 mg/ml on 84nd day.

The antibody titre of egg yolk antibodies was determined by ELISA and it showed the presence of antigen specific antibodies for the specific bacterial pathogen. The titer value is 1:10000. 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.

CONCLUSION:
The present investigation was undertaken to generate antibodies against the bacterial pathogen *clostridium difficile* which causes Antibiotic Associated Diarrhea and Pseudo membranous Colitis in human beings. The cultural and biochemical characteristics of the standard strain was studied. The toxicity of the standard strain was confirmed by EIA Card detector.21 weeks old White Leghorn Chickens were immunized with formalin inactivated whole cell

antigen of *C. difficile* and subsequent booster doses were given periodically at two weeks interval. The eggs were collected and stored at 4ºC.

Purification of IgY antibodies from collected eggs was done according to the method of Polson *et al*., (1980). The concentration of IgY antibodies was estimated 6.98mg /ml on 84th day. The purity of the harvested antibodies was checked by SDS-PAGE and Coomassie brilliant blue staining and it was found to have 180KDa.The titration of antibodies was determined using ELISA where highest titre of more than 1:10000 were observed from 35th day onwards. Thus, the chicken egg yolk anti- *Clostridium difficile* antibodies can be used for the both detection and treatment of Antibiotic Associated diarrhoea by oral passive immunization to humans. Further studies and trials have to be performed.

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