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GENERATION AND CHARACTERIZATION OF CHICKEN EGG YOLK ANTIBODIES AGAINST COMMON PATHOGENS LIKE Canine parvovirus, Canine coronavirus AND *Salmonella typhimurium*.

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Abstract: Specific pathogens have been implicated in acute and chronic gastrointestinal disease due to compositional changes in intestinal microbiota, and lack of immunity leads puppies and adult dogs susceptible to pathogens causing severe gastroenteritis. Gastroenteritis is an inflammation of stomach and intestine and rupture of epithelial cells leading severe bloody diarrhea. The most significant opportunistic pathogens of gastrointestinal tract are parasites, protozoans, viral agents like *Canine Parvovirus, Canine Coronavirus, Canine Distemper virus, Rota virus* with bacterial co-infection by *Salmonella spp, E. coli, Clostridium, Campylobacter* that causes severe gastroenteritis in dogs. *Canine parvovirus 2* (CPV-2) infection is highly contagious viral disease in dogs, which is prevalent all over the world and they are highly fatal. Second most contagious intestinal disease in dogs is *canine coronavirus* infection. The main mode of transmission is by ingestion of infected fecal contaminated material. Salmonellosis caused by *Salmonella typhimurium* is the most common infectious disease in dogs transmitted by fecal matter contamination in foods such as raw meat, chicken, and also occurs more frequently in latent forms. They also cause secondary infection with viral gastroenteritis.

Index Terms: *Canine Parvovirus, Canine Coronavirus, Salmonella typhimurium,* IgY, ELISA.

I. INTRODUCTION

The most common canine intestinal microbes include more than 10 bacterial phyla, Firmicutes, Bacteroidetes, Proteobacteria, Fusobacteria, and Actinobacteria constituting more than 99% of all gut microbiota act as a defending barrier against invading pathogens, aid in digestion, and play a crucial role in the development of the immune system of canines. Specific pathogens have been implicated in acute and chronic gastrointestinal disease due to compositional changes in intestinal microbiota, and lack of immunity leads puppies and adult dogs susceptible to pathogens causing severe gastroenteritis. Gastroenteritis is an inflammation of stomach and intestine and rupture of epithelial cells leading severe bloody diarrhea. Veterinarians care about animal health and also protection of health of animal owners, children and wide public, because many gastrointestinal pathogens in dogs pose a zoonotic risk to humans, including *Campylobacter* spp, *Salmonella spp*, *Clostridium difficile*, *Cryptosporidium* spp, and *E. coli*. According to reports given by veterinarians' *Canine parvovirus, Canine corona virus* rates as first opportunistic pathogens with secondary infection by *Salmonella spp,* compare to other microbes like *E. coli* and *Shigella* causes hemorrhagic gastroenteritis in immune suppressed dogs and susceptible puppies.

Canine parvovirus 2 (CPV-2) infection is highly contagious viral disease in dogs, which is prevalent all over the world. They are highly fatal and have become one of the most significant diseases in canines worldwide, mainly because the virus can survive in harsh environmental conditions for a longer time. The virus causes severe gastroenteritis in animals.

These pathogenic viruses have high morbidity (100%) and frequent mortality up to 10% in adult dogs and 91% in pups (reported in India). *Canine parvovirus* infection enters through oro-nasal route. The most common clinical signs are pyrexia, vomiting, anorexia, and bloody diarrhea. Infected animals have heavy viral shedding in their feces for many weeks. Ingestion of feces contaminated food is the most common way of transmission from animal to animal. Other way of transmission of disease are through aerosols, feces, fomites (dog hair & clothes), vectors (insects & rats). The virus appears within the gastrointestinal tract and propagates in epithelial cells of intestinal crypts and cell degeneration results in destruction of villi. The intestinal wall becomes so damaged that bleeding occurs, allowing bacterial infections to take place. The organism incubates from three days to seven days after exposure before showing signs of illness. The dog shows symptoms of bloody stool within 24 hours of their viral exposure. Puppies die within 48hr if left untreated. The resistance of newborn pups to CPV-2 is passive and depends on the immune status of dam.

Salmonellosis caused by *Salmonella typhimurium* is the most common infectious disease in dogs transmitted by fecal matter contamination in foods such as raw meat, chicken, and also occurs more frequently in latent forms. They also cause secondary infection with viral gastroenteritis. The intestinal wall becomes so damaged, results in an inability to absorb nutrients, causing acute diarrhea. Therefore, puppies die from severe dehydration and shock.

Vaccines have been used to prevent CPV-2 infection for many years. Adult dogs can be vaccinated and boosted yearly. However, the vaccines are, ineffective in young puppies owing to the presence of maternal antibodies in the puppies' blood. As maternal antibody levels wane, the puppies become susceptible to viral infection in contaminated environment. Treatments are given to restore fluid balance, electrolyte balance, and to prevent secondary bacterial infections by routine administration of antibiotics, while the dog's immune system will actively fight the virus, because there is no specific treatment for viral infection.

IgY technology is an alternative method of using IgG antibodies, concerning, laboratory animal welfare, antibodies can be isolated from eggs of immunized hen (serum antibody transfer to egg yolk naturally) without bleeding the animal, usage of IgY avoids unnecessary suffering and leaves the animal for further IgY production during its whole life time, chickens can be maintained under laboratory conditions, they can produce high yield of antibodies compared to mammals. IgY have high affinity and sensitivity. Specific antiviral treatments are lacking and veterinarians are left only to treat the symptoms of disease. Treatment for young puppies gone vain of vaccination and infected animals by co-infection of bacteria can undergo passive immunization by means of oral administration of immune colostrum or immunoglobulins derived from chicken egg yolk (IgY) with probiotics act as an alternative approach for antibiotic usage and for treating lack of vaccination in canines.

II.OBJECTIVES

- Commercially available *Canine parvovirus* (Megavac-P, Ind. Immunol. Ltd) *and Canine coronavirus* (Megavac-CC) vaccines were used as antigen. *Salmonella typhimurium* whole cell antigen was also prepared.
- Generation and characterization of egg yolk antibodies (IgY) from white leghorn chickens immunized with *Canine parvovirus, Canine coronavirus and Salmonella typhimurium.*
- Determination of the specificity and titre of antigen specific IgY in egg yolk by Indirect ELISA. The concentration and purity of antibody (IgY) was determined using SDS PAGE.
- Preparation of spray dried immune Egg yolk powder with commercially available probiotics.

III.REVIEW OF LITERURE

3.1 GASTROINTESTINAL MICRO BIOTA OF CANINES

The pH of the digestive system varies from region to region with more acidic in stomach (pH-3), hence only limited numbers of microbes can thrive in this region, approximately less than $1x10^4$ Cfu/ml. As progress through GI tract digestive enzymes and juices make the pH nearly neutral to slightly alkaline (6-7.5) towards the distal portion facilitating growth of facultative as well as strictly anaerobes including *Streptococci, Lactobacilli, Enterobacteria, Bifidobacteria, Bacteroides* etc. The bacterial population in small intestine reaches up to 10^8 to 10^9 Cfu/ ml, but that of colon is 6.5 pH is profoundly populated with the bacteria, with a total population of 10^{11} to 10^{12} Cfu/ml of contents. In turn the fecal pH lowers to 6 or less. This low pH is due to production of acids because of bacterial fermentation in lower gut (Suchodolski *et al.,* 2011).

3.2 CANINE GASTROENTERITIS

The effect of these antibodies is limited to intestinal contents and surface of the intestinal mucosa; therefore, lactogenic passive immunity is maintained by intake of maternal milk. However, diseases continue to threaten a dog that lacks proper vaccination. The opportunistic pathogens of gastrointestinal tract are parasites, protozoan, viral agents and bacteria causing diarrhoea. Many of these pathogens possess zoonotic risk to humans (Sokolow *et al.,* 2005). The following gastroenteritis causing pathogens are as follows,

3.2.1 VIRAL AGENTS

- Canine Parvovirus

Canine parvovirus is a deadly contagious viral disease that is spread by contact with infected fecal material. The virus is difficult to kill and is easily spread. It attacks the gastrointestinal system, causing fever, lethargy, vomiting, bloody diarrhea, and rapid dehydration. Treatment requires intensive IV fluid and supportive therapy and has a variable rate of success.

- Canine coronavirus

Canine coronavirus is a highly contagious viral infection attacking the gastrointestinal tract. Signs are similar to *parvovirus* infection, except it is generally milder and more effectively treated.

- Canine distemper

Canine distemper is caused by a highly contagious, airborne virus. It affects the dog's respiratory, gastrointestinal, and nervous systems. Early symptoms are those of a "cold" — runny eyes and nose, fever, cough, and often diarrhea. Later in the course of disease there may be nervous twitching, paralysis, and seizures (convulsions). There is no successful treatment.

3.2.2 BACTERIAL AGENTS

Bacterial enteritis can be mild and transient or it can be rapidly fatal. *Salmonella spp, E. coli, Clostridium, Campylobacter* acts as coinfection with viral gastroenteritis in dogs. Bacterial enteritis generally responds to Veterinary treatment.

3.2.3 PARASITES

Various types of parasites often cause diarrhoea. Parasites include hookworms, roundworms, whipworms and tapeworms can all evoke diarrhoea. Hookworms can cause diarrhoea that contains large volumes of blood, or diarrhoea that is black and tarlike. Dogs with a hookworm infection may bleed to death from diarrhea, if it is not treated. Giardia are small single-celled organisms that can attack the cells lining the walls of the intestine. The gastro-enteritis caused by these organisms is easily treated.

3.3 CANINE PARVOVIRUS

There are two types of *canine parvovirus* called *canine minute virus (CPV-1)* and *Canine parvovirus* (CPV-2) causing myocarditis, and gastroenteritis in dogs. This study focuses on CPV-2 which are more severe in dogs and they mutate to form new antigenic types such as CPV-2a, 2b, 2c. CPV-2c has been found as new variant emerging at present (Castro *et al.*, 2010).

3.3.1 GENOME AND STRUCTURE

The small non-structural protein NS2 (25 kDa) formed from a spliced messenger RNA, shares the N-terminal region with NS1. NS2 exist in two forms with slightly differ in C-terminal amino acid sequence. They are expressed in high levels during early infection and each subtype of NS2 exist in approximately equal amount of phosphorylated and in non-phosphorylated form. The non-phosphorylated form shows cytosolic localization, where phosphorylated form shows a uniform distribution in cytosol and in nucleus of infected cells. The Parvovirus capsid lack lipid and carbohydrate (Suikkanen., 2003).

3.3.2 MODE OF INFECTION

Thus, viral DNA replication, expression, and assembly takes place in nuclei of host cell. It is still not clear how the genomes enter the nucleus, but nuclear pore-dependent entry of particles or permeabilization of nuclear membranes have been proposed (Parrish *et al.*, 2010). The NS1 protein has demonstrated to work as an initiator protein for viral DNA replication and as a transcriptional activator of the viral promoters (for capsid protein synthesis), and also for cytotoxic operation in the host cell (Smolander., 2007). Parvoviral DNA replication occurs in host cell nucleus via double strand replicative form and initiated by self-priming mechanism, this synthesis mechanism, is named as “modified rolling hairpin model”.

3.3.3 PATHOGENESIS

The virus infects the germinal epithelium of the intestinal crypts (Hullinger *et al*.,1998). Enterocytes mature here, where they then migrate to the tips of the villi. However, the virus destroys the epithelium, which results in a lack of enterocyte movement and shortening of the villi.

3.3.4 CLINICAL DISEASE

CPV-2 cause enteritis syndrome, is marked by a very characteristic foul smelling, hemorrhagic diarrhea which may or may not contain mucus, and severe vomiting. Animals will become dehydrated, anorexic, and pyrexic. Leukopenia and neutropenia, as well as fecal viral shedding were often observed. Disease is frequently complicated by secondary bacterial infection such as septicemia, endotoxemia caused by *E. coli* (Isogai *et al.*,1989) and *Salmonella spp*. This sepsis can be accompanied by death and can occur as early as two days after the onset of illness. Other enteric pathogens such as *Clostridium perfringens* and *Campylobacter spp Helicobacter spp.*, may contribute to enteritis, necessitating the need for antibiotic therapy during treatment.

3.3.5 EPIDEMIOLOGY

The virus continued to evolve, and by 1980 the original strain of CPV-2 had evolved into type 2a, and again evolving into type 2b by 1984. CPV-2b is predominant in the US and Japan, but both CPV-2b and CPV-2a remain prevalent in Europe and Far East. It is apparent the virus has continued a steady evolution. A third antigenic variant, CPV-2c, was first reported in Italy during the year 2000, in South America during the year 2007, in central Portugal, Europe, Rio de Janeiro, Brazil. etc. In India, at Pondicherry, the present existing strain cpv-2b was found through PCR, RFLP test. (Parthiban *et al*.,2010)

3.3.6 VACCINATION

Vaccination is the best way of preventing CPV infections: Capsid protein: Canine parvovirus empty capsids produced by expression in a baculoviral Vector used for immunization of dogs. (Saliki *et al.,1992).*Attenuated vaccine: Use of modified live vaccine for immunization. (Evermann *et al.,1994).* Peptide vaccine: First Peptide Vaccine Providing Protection against Viral Infection of CPV in infected animals (Langeveld *et al., 1994).* Multivalent vaccine: Three-Year Duration of Immunity for Canine Distemper, Adenovirus, and Parvovirus after Vaccination with a Multivalent Canine Vaccine (Gill *et al.,* 2004*).* DNA vaccine: Cloning of *canine parvovirus* VP2 gene and its use as DNA vaccine in dogs (Gupta *et al*., 2005). RNA Interference used in treatment of *canine parvovirus* (Partridge *et al*.,). Killed vaccine: Heat killed and chemically inactivated *Canine parvovirus* are also used for vaccination. The new treatments available for CPV infection are as follow: Passive immunity, Recombinant human granulocyte colony-stimulating factor, Recombinant bactericidal permeability-increasing protein, Antiviral Protein Polymer (SB73), Specific Oral Antibody, Recombinant feline interferon omega (Lizer, 2008).

3.3.7 DIAGNOSIS AND TREATMENT

Immunochromatography assay (Mossallanejad *et al*., 2008), Immunohistochemistry (IHC), (Evermann, *et al.*, 1994). CPV infection occurs concurrently along with other viral, bacterial pathogen, parasitic infection showing the same hemorrhagic diarrhoea symptoms, thus laboratorial diagnosis is essential to confirm CPV or parasitic infection to proceed for further treatment (Castro *et al*., 2010).

3.4 CANINE CORONAVIRUS

3.4.1 GENOME AND STRUCTURE

This domain is followed by a triple-membrane-spanning domain and a large carboxyl-terminal domain inside the envelope. Although the major immunological role has been attributed to the S protein, antibodies to the M protein of MHV can neutralize viral infectivity but only in the presence of complement. The nucleocapsid protein N is a basic phosphoprotein of 50–60 kDa that binds to virion RNA, providing the structural basis for the helical nucleocapsid N plays a role in viral RNA synthesis and interacts with M protein leading to the formation of virus particles. Additional ORFs encoding nonstructural proteins have been recognized in CoV genomes. The functions of such genes are in most cases unknown and most of them are not essential for virus replication, but may play a part in virulence and host range (Pratelli *et al*., 2011)

3.4.2 MODE OF INFECTION

Canine coronavirus is a highly contagious viral infection attacking the gastrointestinal tract. Signs are similar to parvovirus infection, except it is generally milder and more effectively treated. A *canine coronavirus* infection is a highly contagious intestinal disease of dogs all around the world. The main mode of transmission is by ingestion of infected fecal material. The symptoms of a *canine coronavirus* infection vary slightly from *canine parvovirus* as it invades the rapidly growing cells of the intestinal lining causing nausea, lack of appetite, vomiting and diarrhea. The diarrhea associated with *Coronavirus* usually lasts several days with low mortality but *Parvovirus* and *Coronavirus* cause the same appearing diarrhea with an identical odor. A more serious complication of *canine coronavirus* occurs only when the dog is also infected with *canine parvovirus*, and the two infections can be indistinguishable without lab test.

3.4.3 PATHOGENESIS

The factors regulating the course of the natural diseases caused by enteric CCoVs are not well understood. The virus appears to replicate primarily in the villus tips of the enterocytes of the small intestine causing a lytic infection followed by desquamation and shortening of the villi and resulting in diarrhoea 18–72 h post infection. Production of local IgA restricts the spread of the virus within the intestine and arrests the progress of the infection. Therefore, infected dogs may shed virus for as long as 6 months after clinical signs have ceased. These viruses are very milder and also infection be there for longer time. They show symptoms similar to CPV. But these virus infections become severe only when there is coinfection of CPV. And other secondary infection due to *Salmonella spp, Clostridium, Camphylobacter* etc. causes severe bloody diarrhoea. (Pratelli *et al.*, 2011)

3.4.4 EPIDEMIOLOGY

Nevertheless, the disease caused by CCoV has not been adequately investigated, and the role it plays in canine enteric illness is not established in detail. Since its first isolation in 1971, CCoV has evolved, and in the last two decades researchers have focused on the genomic variability of CCoV strains, identifying new genotypes/types. A possible explanation is that under natural conditions homologous recombination events between highly homologous CoVs, such as CCoV and FCoV, occur frequently. This means that a frequent interspecies circulation either of FCoVs to dogs or of CcoVs to cats may occur, since mixed infections are required to give rise to recombination events. CCoVs type 1 and type 2 were found to be common in an Australian animal shelter with CCoV type 1 being prevalent. CCoVs have also been found in Western European dog CCoVs have also been found in Western European dog (Pratelli *et al*., 2011).

3.4.5 VACCINATION

Inactivated form of CCV vaccine can significantly reduce not only virus replication. But if vaccination is ineffective the susceptible dogs are infected.

3.4.6 DIAGNOSIS AND TREATMENT

CCoVs are not easily disentitled from those associated with other enteric pathogens such as CPV2 or canine rotavirus and canine adenovirus. The diagnostic techniques employed for the detection of CCoVs in fecal samples include electron microscopy (EM), virus isolation (VI) in cell cultures, and biomolecular analysis. EM examination of negatively stained fecal suspensions and immune electron microscopy are rapid procedures for detecting coronavirus and appear to be valuable diagnostic tools. However, *coronavirus-like* particles in intestinal contents often resemble coronaviruses, and EM examination required specialized laboratories and technicians. VI is the most commonly used technique for diagnosis of CCoVs infection but is more complex, more time-consuming and less sensitive than other methods have been identified as the gold standard because of the improvement in both sensitivity and specificity when compared to conventional methods. the feces of infected dogs, based on the TaqMan technology. There is no specific treatment for viral infection. Electrolyte is given and secondary symptoms are treated (Pratelli *et al.*, 2011).

3.5 SALMONELLA TYPHIMURIUM

3.5.1 GENOME AND STRUCTURE

The novel, never previously used name "*Salmonella enterica"* has therefore been proposed as a replacement for the name *"Salmonella choleraesuis."* According to this system of nomenclature, *"Salmonella typhimurium*" would be renamed *"Salmonella enterica* serotype Typhimurium." Although this proposal was not formally adopted by the International Committee of Systematic Bacteriology, these names have become accepted for use by the World Health Organization and in publications of the American Society for Microbiology. Most medical laboratories continue to report clinically familiar names, such as *Salmonella typhimurium* or *Salmonella* serotype Typhimurium. (Hohmann *et al.*, 2011)

3.5.2 MODE OF INFECTION

More than 95% of cases of *Salmonella* infection are foodborne, and salmonellosis accounts for 30% of deaths resulting from foodborne. After *Campylobacter*, *Salmonella* is the most commonly isolated bacterial pathogen, when laboratory diagnosis of diarrhea is sought. Acquisition of *Salmonella* from pets (e.g., reptiles and birds), direct personal contact, nosocomial transmission, waterborne transmission, and contaminated drugs and solutions are fewer common modes of transmission. In the late 1990s, *S. typhimurium* serogroup B and *Salmonella enteritidis* serogroup D were the most frequently isolated serotypes (Hohmann *et al*., 2011)

3.5.3 PATHOGENESIS

There is clearly room for improvement in the quality management and manufacturing practices of the meat industry for greyhounds. The dog owners and other kennel personnel should be made aware of the dangers involved in feeding 4-D meat products. Preventive measures such as proper cooking, chemical treatment, and proper handling and storage of meat should also be brought to the attention of people in the industry. The increase in resistance of *Salmonella* to antimicrobial agents has been a major problem for practitioners of veterinary and human medicine. (Chengappa *et al.*,1993)

3.5.4 DIAGNOSIS AND TREATMENT

Salmonella (22.2%) were resistant to amoxicillin, 20 (50%) to ampicillin/clavulanic acid, 9 (22.5%) to apramycin, 1 (2.8%) to cefoxitin, none to ceftriaxone, 2 (5%) to cephalothin, 21 (52.5%) to chloramphenicol, none to ciprofloxacin, 2 (5%) to gentamicin, 2 (5%) to kanamycin, 17 (42.5%) to nalidixic acid, 4 (10%) to nitrofurantoin, 14 (38.9%) to streptomycin, 15 (37.5%) to sulfamethoxazole/ trimethoprim and 31 (77.5%) to tetracycline. the canine Salmonella isolates were susceptible to ceftriaxone and ciprofloxacin. Thus, antibiotics are given for treatment. (Tsai *et al.*, 2007).

3.6 IGY –TECHNOLOGY

Antibodies presently available for research, diagnostic and therapies are mostly mammalian monoclonal or polyclonal antibodies. Traditionally, bigger animals such as horses, sheep, pigs and also rabbits and guinea pigs, were used for the production of polyclonal antibodies. Nowadays, most frequently chosen mammals for polyclonal and monoclonal antibody production are rabbits and mice (blood) respectively, where the major problem of monoclonal antibody production is that some antigens are weakly or not at all immunogenic for mice, and antibody production and purification rate is very low. And also bleeding makes distress to animals. Recently, considerable research has focused on the use of IgY as an alternative to mammalian antibodies for several applications, including for immunotherapeutic applications, especially for the oral passive immunization against various bacteria and viruses, immunodiagnostic and immunoaffinity purification purposes. The use of IgY offers several advantages over polyclonal antibodies produced in mammals, including providing a much more hygienic, cost efficient, convenient, humane and plentiful source of antigen-specific antibodies. (Schade *et al.,1996).*

3.6.1 PRODUCTION OF IGY

The presence of yolk antibodies should be checked two weeks after the second immunization. When the antibody titer decreases, booster immunizations can be given during the whole laying period. A laying hen produces five to six eggs per week. Average volume of egg yolk (15 mL) contains 50–100 mg of IgY, of which 2 to 10 % are specific antibodies. Compared to the production of polyclonal antibodies in mammals, this is significantly greater yield that only large mammals, such as a horse, can reach (Narat *et al*., 2003).

3.6.2 PURIFICATION OF IGY

Egg yolk contains large amount of lipids. To avoid problems in immunoassay isolation of IgY from the egg yolk should be carried out as a final step of IgY technology. Though they do not bind to protein A and G, which is a popular method for isolation of IgG, there are many other methods enabling a successful purification Frequently cited methods are affinity purification, ammonium sulphate or PEG precipitation. (*Narat et al., 2003).*

3.6.3 STRUCTURE AND CHARACTERISTICS OF AVIAN IGY AND MAMMALIAN IGG ANTIBODIES

Avian serum immunoglobulins were classified as IgG-like immunoglobulins that are transferred to the egg yolk, called Ig Y. Now IgY is recognized as a typical low-molecular-weight serum antibody of birds, reptiles, amphibia and lungfish, and as an evolutionary ancestor of IgG and IgE antibodies (Table:1) that are unique to mammals only. General structure of IgY molecule is the same as of IgG with 2 heavy (H)chains with a molecular mass of 67–70 kDa each and two light (L) chains with the molecular mass of 25 kDa each the major difference is the number of constant regions (C) in H chains: IgG has 3 C regions (C1 –C3), while IgY has 4 C regions (C1 – C4). One additional C region with two corresponding carbohydrate chains has a logical consequence in a greater molecular mass of IgY compared to IgG *i.e.* 180 and 150 kDa, respectively. IgY is much less flexible than IgG due to the absence of the hinge between C1 and C2, which is a unique mammalian feature. There are some regions in IgY (near the boundaries of C1–C2 and C2–C3) containing proline and glycine residues enabling only limited flexibility. IgY has isoelectric point 5.7–7.6 and is more hydrophobic than IgG. Regarding the relatively high core body temperature of chickens, which is 41 °C, it is not surprising, that half-life time of IgY is in months and that they retain their activity after 6 months at room temperature or for one month at 37 °C.

3.6.4 IGY AS AN ALTERNATIVE TO MAMMALIAN IGG

The use of chickens for the production of polyclonal antibodies provides several advantages over the traditional method of producing antibodies in mammals. In contrast to mammalian serum, egg yolk contains only the single class of antibody, IgY, which can be easily purified from the yolk by simple precipitation techniques. (Nolan *et al.*, 2004).

3.6.5 Application of IgY in veterinary fields

Several studies have demonstrated that chicken IgY has similar affinity, and sensitivity to mammalian IgG. Indeed, antibody production of hen is approximately 17-35 g of total IgY/chicken/year of which1-10% of antibodies are specific to antigen. IgY based Immunoassay are used to measure the concentration of protein, peptide, via ELISA, RIA and other assay in clinical chemistry and basic research. IgY are also used in Immunohistochemistry for detecting antigens of viral, bacterial, plant, and animal origin. To

assess the incidence of intestinal parasite in domestic animals (Schade *et al*.,2007). They do not cross react with mammalian IgG. IgY used in various techniques such as, Immunotherapy, Monoprophylaxis for several bacterial and viral infections in animals. Thus, egg yolk immunoglobulin is a novel tool in veterinary field.

3.7 PROBIOTICS

Administration of *Lactobacillus acidophilus* increased granulocyte phagocytic activity. Growing puppies that received *Enterococcus faecium* for 20 week showed increased plasma IgA concentrations compared to the control group (Suchodolski *et al.*, 2010). yeast specifically *Saccharomyces cerevisiae* contain *ß*-glucans on cell walls which are proposed to enhance immune system function. Bacillus strains are known for optimizing food utilization. *Bifidobacterium bifidum* is critical for aiding young animals in the production of LAB and producing enzymes for cell development and cell repair. The commercially available products have Fructo-oligosaccharide (FOS) is a natural source of inulin, a carbohydrate made up of fructose polymers. The FOS cannot be metabolized by monogastric, which lack the enzyme inulin's. The FOS passes to the lower intestine where it is fermented by LAB such as *Bifidobacterium*. Gram negative bacteria, such as *Escherichia coli (E. coli)*, are unable to grow on FOS; this creates a natural selection process which promotes beneficial bacteria in the lower intestine.

IV.MATERIAL AND METHODS

4.1 EXPERIMENTAL ANIMAL

White Leghorn Laying chickens, 24 weeks old, 3 in no's were bought from LK Poultry farm, Palladam. The hens were kept in isolated cages at the animal house and provided regular food and water.

4.2 ANTIGEN SOURCE

4.2.1 CANINE PARVOVIRUS (CPV)

Canine parvovirus antigen was obtained in the form of "Canine parvovirus Vaccine Inactivated IP (Vet)", a commercially available product of Indian Immunological Limited. This vaccine contains tissue adapted strain of Canine parvovirus grown on A-72 cell cultures and inactivated with beta–propiolactone. Aluminum hydroxide gel is used as an adjuvant. And the vaccine contains approximately 10^3 viral particles per ml.

4.2.2 CANINE CORONAVIRUS (CCV)

Canine Coronavirus antigen was obtained in the form of "Canine Coronavirus Vaccine Inactivated IP (Vet)", a commercially available product of Indian Immunological Limited. This vaccine contains inactivated tissue culture Canine Coronavirus antigen grown on a continuous cell line and suitably inactivated with beta–propiolactone. Aluminum hydroxide gel is used as an adjuvant. And the vaccine contains approximately 10^3 viral particles per ml.

4.2.3 SALMONELLA TYPHIMURIUM

Salmonella typhimurium was obtained from MTCC (Microbial type culture collection).

4.2.3.1 MICROSCOPIC EXAMINATION

Isolated bacterial colonies were verified microscopically by gram staining. The prepared smear was air dried and heat fixed. Crystal violet was flooded over the smear for one minute and drained. After washing, Gram's iodine was added and left for one minute. The smear was then washed in 95 % (v/v) ethanol. It was counter stained with Safranin for 30 sec. After air drying, the smear was examined under microscope with oil immersion. And motility test of bacteria was examined using hanging drop method.

4.2.3.2 MORPHOLOGICAL AND BIOCHEMICAL CHARACTERIZATION

Standard culture was studied for its colony morphology and biochemical character.

4.2.3.3 BACTERIAL ANTIGEN PREPARATION

Salmonella cells were grown in 400ml of Tryptic Soy broth for overnight at 37°C with continuous shaking at 100 rpm. Bacterial cells were harvested by centrifugation for 20 minutes at 10,000 rpm at 4°C. They were washed twice with phosphate buffered saline (PBS-pH-7.2) and resuspended with same buffer. Resuspended bacterial cells were adjusted to McFarland No: 1 standard. Further they are inactive by heat killing at 80°C for 30 minutes. (Xiao-liang Li *et al*., 2006). The sterility of antigen preparation was tested. Heat killed cells were spread plated on Tryptic Soy Agar medium and incubated at 37°C for overnight. After conformation of no growth on medium, the prepared antigen was taken for immunizing chickens.

4.3 IMMUNIZATION OF ANIMAL

Antigen per chicken was immunized intramuscularly on four sites of breast muscles. The dosage varies based upon the antigen usage.

Table: 1 Immunization schedules

ANTIGEN	DOSAGE SCHEDULE	DAYS	ANTIGEN DOSAGE	ADJUVANT	SITE OF INJECTION
CPV	1st dose	0th	1 ml ~ (10^3 viral particle/ml of vaccine)	Aluminum hydroxide	Intramuscular
	1st Booster	15th			
	2nd Booster	30th			
	3rd Booster	45th			
CCV	1st dose	0th	1 ml ~ (10^3 viral particle/ml of vaccine)	Aluminum hydroxide	Intramuscular
	1st Booster	15th			
	2nd Booster	30th			
	3rd Booster	45th			
Salmonella typhimurium	1st dose	0th	1 ml ~ (10^9 cells/ml)	-	Intramuscular
	1st Booster	15th			
	2nd Booster	30th			
	3rd Booster	45th			

4.4 COLLECTION AND STORAGE OF EGGS

The eggs were collected from first day of immunization and labelled with an identification number, date using a waterproof pen. The collected eggs were refrigerated at 4ºC until the isolation of the immunoglobulins. Yolk antibodies are stable *in situ* in the refrigerator for at least 6 months.

4.5 SEPARATION OF EGG YOLK FROM WHITE

The egg yolk was separated from white and was washed with water to remove albumins. The yolk was rolled on tissue paper and the yolk membrane was punctures 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 is obtained from an average sized egg.

4.6 PURIFICATION AND CONCENTRATION OF EGG YOLK ANTIBODIES

Separated yolk from the eggs of immunized hens, were mixed (double the volume of yolk) 100mM phosphate buffer, pH 7.6 and mixed thoroughly with a glass rod. 3.5% (w/v) polyethylene glycol (PEG 6000) was added and mixed until the PEG completely dissolved. The sample was centrifuged at 11000 rpm for 20 min at room temperature. A cotton wool (absorbent-type) plug was firmly placed at the base of a funnel, and the supernatant was filtered through it. The lipid fraction is trapped by the cotton wool. The filtrate must be a clear yellow liquid and not milky in appearance; if the filtrate is not clear, the filtration step is repeated. The filtrate volume was recorded, and the PEG concentration was increased (it is already at 3.5%) to 12% (*i.e.* add 8.5%). The PEG was dissolved completely by mixing. The suspension was centrifuged at 11000 rpm for 20 min at room temperature. The remaining supernatant was discarded. The pellet was dissolved in a volume of phosphate buffer equal to the egg yolk volume. PEG was again added to a final concentration of 12% (w/v) (1.2g), mixed thoroughly, and centrifuged at 11000 rpm for 20 min at room temperature. The supernatant was discarded and the final pellet containing the antibodies was resuspended in 800 micro liters of the phosphate buffer, and taken to dialysis.

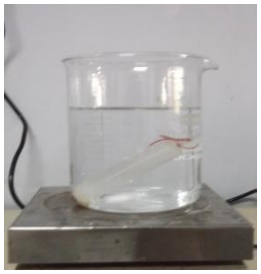
Maintenance of chicken

Canine Parvovirus

Canine Coronavirus

Immunization

Egg collection and storage



Yolk separation and purification

Semi purified aliquots

DEAE cellulose column

Figure:1- Immunization and purification of IgY.

4.7 DIALYSIS

4.7.1 ACTIVATION OF DIALYSIS BAG

The tubing was cut into pieces of required and convenient length and allowed to boil for 10 minutes in a large volume of sodium bicarbonate buffer. The tubing was rinsed thoroughly in distilled water. Again, it was allowed to boil in 1mM EDTA (pH 8.0). The tubing was cooled down and stored at 4°C in 50% ethanol. Care was taken that the tubing was thoroughly submerged. After this step, the tubing was handled with gloves. Before use, the tubing was washed inside and outside with distilled water.

4.7.2 DIALYZING YOLK ANTIBODIES

One end of the dialysis tube was tied with a thread and ensured it was leak-proof. The purified egg yolk antibodies were transferred to an active dialysis bag and tied after including some air and twisting the open end of the dialysis bag. The bags containing pooled contents were dispensed into saline for overnight. The setup was continuously stirred by means of a magnetic stirrer and the temperature was maintained at 4^0C. The dialysis was changed to phosphate buffer saline and carried out over 3 hours incubation by stirring. The contents were dispensed into tubes after dialysis (plate:1h). The final concentration increased maximum to 2ml. (Schade *et al., 2011*). After purification and dialysis, the samples in aliquots were stored at 4˚C. The IgY was taken for further purification using DEAE cellulose Ion Exchange Column Chromatography.

4.8 DEAE CELLULOSE ION EXCHANGE COLUMN CHROMATOGRAPHY

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

4.8.1 PACKING OF CHROMATOGRAPHY COLUMN

For purification of immunoglobulin, the column size 2x30 cm was used. The burette was first cleaned well and it was packed first with glass wool to form an even bed and a rubber tube with pinch-cork was attached to the tip of the burette. The column was fixed to stand in vertical position. The DEAE slurry was poured into the column along the sides to avoid air bubbles and was allowed to settle. Once the column was set, it was equilibrated with 25mM phosphate buffer, pH 8.0 (till the out flow of buffer showed pH 8.0).

4.8.2 SAMPLE APPLICATION AND ELUTION

Once the column was equilibrated (25mM phosphate buffer, pH 8.0) the level of buffer in column was allowed to run down to the matrix and the outlet was closed. The immunoglobulin IgY sample layered on the top of the column and was allowed to run till all the sample had entered the bed. Then a continuous constant flow of 25mM phosphate buffer was maintained until all the protein came out. IgY was eluted with 250mM phosphate buffer pH 8.0. These eluted IgY fractions are checked for its concentration and purity using SDS PAGE.

4.9 ESTIMATION OF TOTAL PROTEIN

The dialyzed egg yolk antibodies were estimated for total protein concentration. Egg yolk antibodies were diluted with sterile distal water in 1:40 ratio (100 µl of sample + 3900 µl of distal water). Following OD taken at 280 nm and 260 nm. This value gives the total protein concentration (dialyzed egg yolk antibodies) in mg/ml (Spectrophotometric determination of protein concentration, Warburg Christina).

4.10 ESTIMATION OF TOTAL IGY

The dialyzed egg yolk antibodies were estimated for total IgY concentration. Egg yolk antibodies were diluted with sterile distal water in 1:10 ratio (300 µl of sample + 2700 µl of distal water). Following OD taken at 280 nm. This value gives the total IgY concentration (dialyzed egg yolk antibodies) in mg/ml (Determination of total IgY concentration, Gallus Immunotech Inc). The eggs collected from first day of immunization are taken in random, purified and estimated for total protein and total IgY concentration.

4.11 SLIDE AGGLUTINATION TEST

It is a qualitative test of chicken egg yolk antibody and bacterial antigen. A Greece free glass slide was taken. On slide 10µl of antibody (chicken egg yolk antibody raised against *Salmonella typhimurium*) was mixed with 10µl of antigen (heat killed *Salmonella typhimurium* antigen) using applicator stick. Agglutination reaction can be detected within minutes by comparing with control (replaced antibody with saline).

4.12 MICRO AGGLUTINATION TEST

Micro agglutination (MAT) used to quantify the titre of the specific antibody. MAT was done in micro plate containing 96 "U" bottom wells. The bacterial antigens were prepared and inactivated by heat killing treatment and the antigen's turbidity was adjusted to 0.5 McFarland standard using sterile PBS (pH-9.2). Following antigens were stained with 1% crystal violet solution and allowed for incubation at room temperature for 30 minutes. The cells were harvested and washed thrice with sterile PBS (pH-9.2) and the turbidity adjusted to its initial McFarland concentration.

4.13 TITRATION OF SPECIFIC ANTIBODIES BY ELISA

The immunological specificity of IgY titre elaborated against *Canine parvovirus* and *Corona virus* antigen was examined quantitatively by enzyme-linked immunosorbent assay (a modified method of Pokorova *et al.*, 2000). In brief, wells of Microtiter plates were coated with 100 µI of antigen solution appropriately diluted with 0.05 M carbonate buffer (pH 9.6). After overnight incubation at 4°C, the plates were washed, and 200µ1 of PBS (pH 7.4) containing bovine serum albumin (1% in PBS) was added to the wells in order to block the uncoated surface. After being blocked each well was washed three times with 200 µL of PBS (0.85% NaCl, 0.01 M phosphate buffer, pH 7.2) - Tween (containing 0.05% Tween 20), and IgY from immunized hens at different time intervals was applied to the well in duplicate for reaction with the antigen for 1 hours at 37°C. After each well was washed again with 200µL of PBS Tween, 100µL of horseradish peroxidase-conjugated rabbit anti chicken IgG (Sigma Chemical Co.) diluted (1:1000) with PBS Tween was added to each well, and the plate was incubated at 37°C for 2 hours. Each well was washed again with 200 µL of PBS-Tween, and then 100 µL of TMB solution with H_2O_2 (Genei Pvt. Ltd., Bangalore). The reaction was stopped after 20 min with 4N H_2SO_4 (50µl per well), and the intensity of color developed was measured at 450 nm with a microplate reader.

4.14 PURITY OF CHICKEN EGG YOLK ANTIBODY USING-SDS PAGE

The IgY fractions (antibody samples of all three antigen) from other proteins were separated by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) according to Laemmli, 1970 with a 4% stacking and 10% separating gel. The samples were taken (20µl) and treated with an equal amount of sample treatment buffer consisting of 5% mercaptoethanol, 2% SDS, 0.005% bromophenol blue dye in 0.05M Tris buffer pH 8.0. The samples were loaded into sample wells along with a known molecular weight marker (Low and Mid-range markers, Genei Pvt. Ltd, Bangalore) and the electrophoresis was performed at 100V current in a Tris glycine buffer system (Plate:). When Bromo phenol blue dye reached at the bottom of the gel, the electrophoresis was stopped. The gel was removed and was stained with Coomassie brilliant blue R-250 for 30 minutes. The gel was rinsed with distilled water and detained. The molecular weight of the proteins resolved estimated in comparison to the molecular weight markers.

4.15 PREPARATION OF SPRAY DRIED EGG YOLK POWDER

Immunized egg yolk antibodies (of all three antigens -*Canine Parvovirus*, *Canine Coronavirus*, *Salmonella typhimurium*) from 50th day after three boosters, were collected. The yolks are separated and pooled out aseptically (Separately for each antigen). The yolks are diluted with equal volume of sterile distal water to facilitate the operation, (IGNARIO., 2007). Relatively high temperatures are needed for spray drying operations. But the typical surface temperature of a particle (egg yolk) during the constant drying zone (Temperature of feed) was 60˚C.The sprayed product and drying air flow go in the same direction. The dried material

was separated from air in a cyclone separator, and the yolk powder was collected. Spray dried yolk powder can be given orally as passive immunization therapy for dogs following efficacy study on *in-vitro* and *in-vivo* neutralization test of IgY antibodies is yet to be done in further studies.

V.RESULT

5.1 MICROSCOPIC EXAMINATION

5.1.1 GRAM STAINING

Standard culture was stained by gram staining technique. Microscopic view under oil immersion showed it as gram negative, pink rods.

5.1.2 MOTILITY TEST

Standard culture was tested for its motility using hanging drop method. And culture showed motility.

5.2 MORPHOLOGICAL AND BIOCHEMICAL CHARACTERIZATION

Standard culture was inoculated on Bismuth Sulphide agar medium (selective media for *Salmonella typhimurium*). It showed colony morphology as circular smooth black, flat dry colonies surrounded by a black-brown zone with metallic reflection and the biochemical characters are studied and tabulated.

Table:2- The biochemical characterization of standard culture

Biochemical test	Culture
Indole	+
Methyl red	-
Voges-Proskauer	-
Citrate utilization	+
Triple sugar iron agar	K/A/G, H_2S
Glucose	A/G
Lactose	-
Sucrose	-
Mannitol	A/G
Oxidase	-
Catalase	+

5.3 GENERATION AND PURIFICATION OF CHICKEN EGG YOLK ANTIBODIES

Twenty-four-week-old white leghorn chicken were immunized intramuscularly by 1ml of Canine Parvovirus and Canine Corona viral vaccine (contain ~10^3TCID/ml of vaccine) and 1 ml of *Salmonella typhimurium* antigen (10^9 cells/ml). The antibody was generated in immunized chicken by periodic booster dosage, whereas objective study of raising antibody in egg yolk results were as follows. After immunization followed by booster dosages, eggs were collected purified (Schade *et al.*, 2011). The precipitate was dialyzed. The egg yolk antibodies were further purified by DEAE cellulose ion exchange column chromatography and different fractions were collected. The (IgY) antibody purity and concentration were detected by SDS PAGE (3-4thfraction collection). Specific IgY titre were determined by ELISA and Micro agglutination test.

5.4 ESTIMATION OF TOTAL PROTEIN

The dialyzed egg yolk antibodies were estimated for total protein concentration. Egg yolk antibodies were diluted with sterile distal water in 1:40 ratio (100 µl of sample + 3900 µl of distal water). Following OD taken at 280 nm and 260 nm. The protein concentration in egg yolk was increased and reached a maximum of 57.4 mg/ml as seen.5.5 Estimation of total IgY Protein content of the eluted IgY antibodies were estimated by diluting Egg yolk antibodies with sterile distal water in 1:10 ratio (300 µl of sample + 2700 µl of distal water). Following OD taken at 280 nm. The IgY concentration in egg yolk was increased during the immunization period and reached a maximum of 42.3 mg/ml. The amount of IgY/ml of yolk was significantly higher when the chicken received

booster injections at regular intervals.

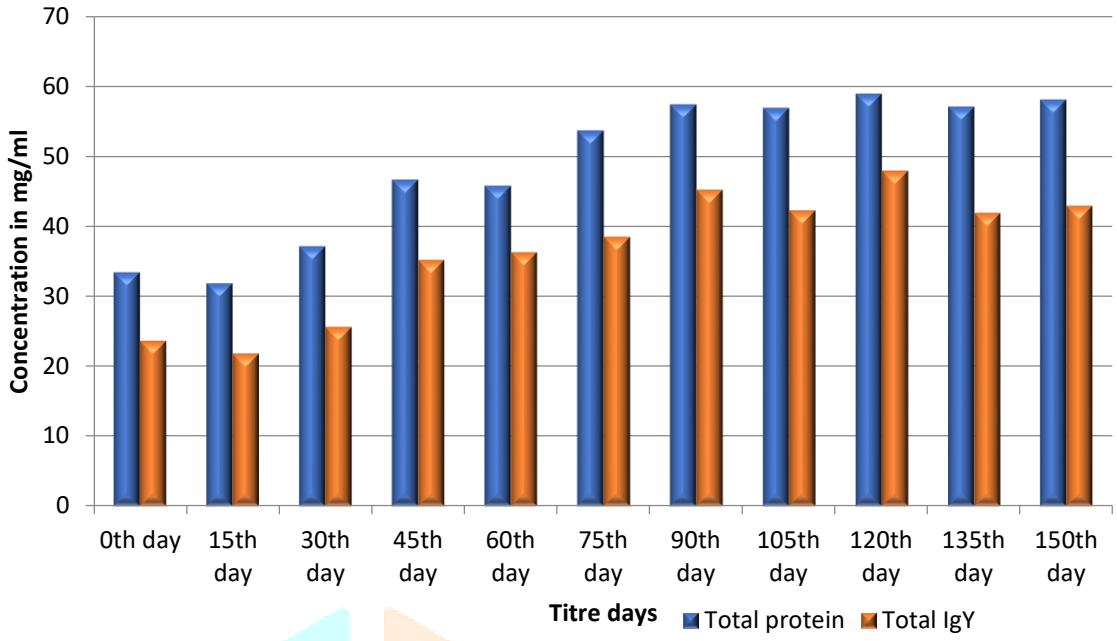


Figure:2- Estimation of the total protein and total IgY of anti-CPV immune eggs

Figure:3-Estimation of the total protein and total IgY of anti-*S. typhimurium* immune eggs

5.6 SLIDE AGGLUTINATION TEST

Qualitative determination of the presence of chicken egg yolk antibody against whole cell bacterial antigen (*Salmonella typhimurium)* was determined by slide agglutination. Visible clumping appeared within 30 sec to 1 min which shows the presence of specific IgY binding with bacterial receptors.

5.7 MICRO AGGLUTINATION TEST

Quantitative titre of the specific antibody was determined by micro agglutination test. The column purified egg yolk antibodies derived from immunized hen were added to the agglutination assay plate, which were coated with each antigen. The IgY antibodies bound specifically with the components of the antigen. The binding pattern of IgY was visualized by agglutination. The minimum agglutination concentration of IgY against whole cell antigen of *S. typhimurium* was found to be 1:1280. Agglutination of these antigen on assay plate confirmed the derived IgY were specific to antigen used.

Table: 3 Micro Agglutination Titre of Anti-*Salmonella typhimurium* IgY

Days of immune eggs	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560
0	+	+	-	-	-	-	-	-	-
15	+	+	+	+	-	-	-	-	-
30	+	+	+	+	+	+	-	-	-
45	+	+	+	+	+	+	+	+	-
60	+	+	+	+	+	+	+	+	-
75	+	+	+	+	+	+	+	+	-
90	+	+	+	+	+	+	+	+	-
105	+	+	+	+	+	+	+	+	-
120	+	+	+	+	+	+	+	+	-
135	+	+	+	+	+	+	+	+	-
150	+	+	+	+	+	+	+	+	-

5.8 TITRATION OF SPECIFIC ANTIBODY BY ELISA

The specificity and dynamics of antibody raised against Canine parvovirus and Coronavirus after periodic boosting the immune system of chickens were studied. The purified samples are taken for analyzing the titre of antibodies raised in egg yolk using Indirect ELISA method. The level of specific antibody raised were measured at an optical density of 450 nm. The specific antibodies for started to appear in egg yolk two weeks after immunization began and reached high titre on 60th days later which remained stable for at least the 150th day period of observation. The titre values were significantly increased after immunization with each antigen. A peak titre of more than 1:10000 were observed in immunized chicken egg yolk against respective antigens.

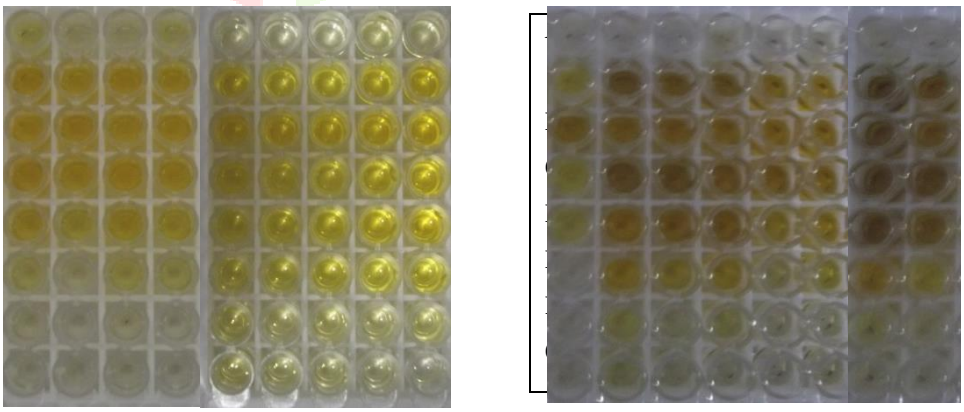


Figure:5-Quantitative estimation of antibodies fraction from egg yolk by ELISA. 1-Canine Parvovirus, 2-Canine Coronavirus. A-blank, B, C, D-(sample), E-1:10, F-1:100, G-1000, H-10000 (sample dilution).

Figure: 6-Determination of antibody titre in immune eggs using indirect ELISA

5.9 PURITY OF CHICKEN EGG YOLK ANTIBODY- SDS PAGE

The purity of chicken egg yolk antibodies and its molecular weight were determined by Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis (SDS-PAGE) using 10% gel according to the method of Laemmli (1970). The high molecular weight protein (180KD) showed the purity of IgY.

180KDa

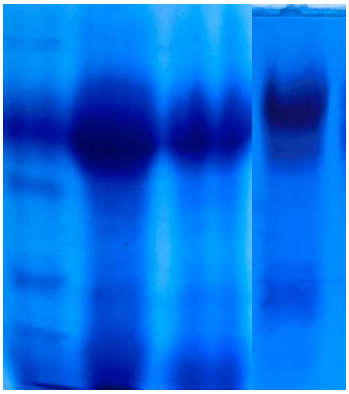
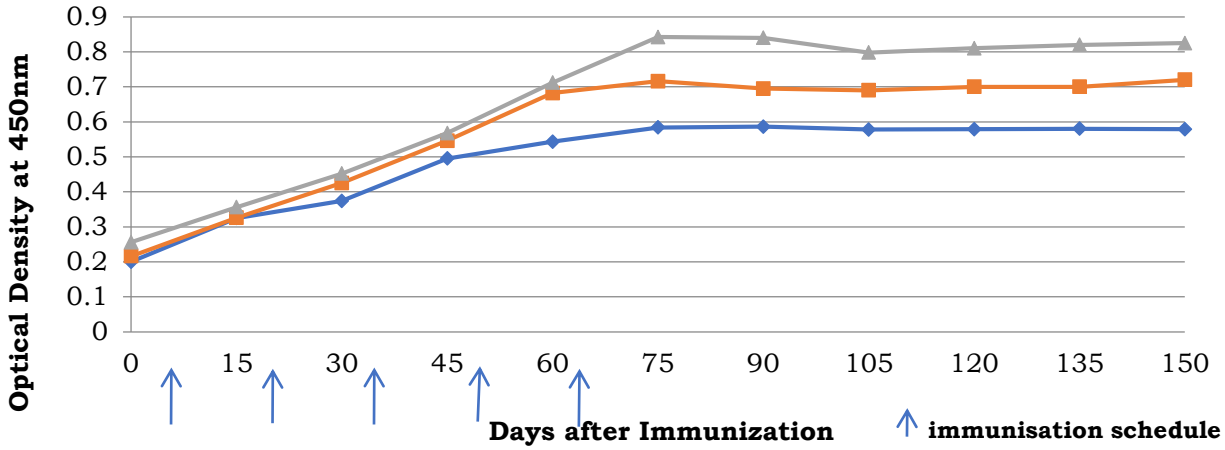


Figure:7-The high molecular weight band
(180 K Da) shows IgY antibody
1-marker,2-CPV,3-CCV

5.10 PREPARATION YOLK POWDER WITH PROBIOTICS:

Spray dried yolk powder prepared by pretreating yolk with equal volume of sterile distal water because, higher the dilution easier the operation and higher the yield. The egg yolk powder was yellow in color, and homogenously dry substance. The protein content before and after spray drying may have some amount of protein denaturation which are not considered as significant loss. 15 ml (1 egg yolk) of yolk can give approximately 3-4g of spray dried yolk powder. The spray dried yolk powder of all antigen is blended with probiotics (commercially available product for dogs contains *Lactobacillus acidophilus, and Enterococcus faecium*. Efficacy study of specific IgY in neutralization test is yet to be done on further studies.



VI.DISCUSSION

The present study is to raise chicken polyclonal antibody (IgY) against gastrointestinal pathogens *Canine Parvovirus, Canine Coronavirus, Salmonella typhimurium* which causes severe gastroenteritis in dogs. *Canine parvovirus, Coronavirus* antigen (contains 10^3 viral particle/ml with Aluminum hydroxide as adjuvants) (Pokorova *et al.*, 2000), and *Salmonella typhimurium antigen* (10^9 cells/ml) were prepared and immunized in white leghorn chickens intramuscularly on breast muscles. The eggs were collected from first day of immunization, followed yolk separated and purified. (Schade *et al*., 2011). The purified yolk antibody of all three antigens was estimated for total protein and total IgY concentration. (Spectrophotometric determination of protein concentration, Warburg Christina and Determination of total IgY concentration, Gallus Immunotech Inc) The protein concentration in egg yolk was increased and reached a maximum of 57.4 mg/ml. And total IgY concentration in egg yolk was increased and reached a maximum of 42.3 mg/ml, both was found to remain constant till 150th day of study.).

Qualitative determination of chicken egg yolk antibody against whole cell bacterial antigen (*Salmonella typhimurium)* by slide agglutination showed visible clumping appearance Quantitative determination of IgY against *Salmonella typhimurium* was found by micro agglutination. There was good agglutination (cottony fluffy appearance at the bottom of wells) up to 1:1280, this shows frequent booster dosage, increase the titer value. Dynamics of specific IgY were determined by indirect ELISA method. And it showed a highest titer up to 1:10000. This shows periodic boosting immune system of chickens, raises specific IgY antibody concentration in egg yolk, from 60th day it remains constantly till 150th day. After DEAE Cellulose Ion Exchange Column Chromatography IgY fractions are checked for its concentration and purity using SDS PAGE. Anti-CPV antibody, anti-CCV

antibody, anti-*S. typhimurium* antibody showed a high molecular weight of 180 K Da. The present study showed that chicken polyclonal antibody (IgY) can be raised against Canine parvovirus, coronavirus and *Salmonella typhimurium.* The dynamics of specific IgY can be raised by subsequent booster dosage. In vitro and in vivo neutralization test of IgY antibodies is yet to be studied. Purified antibodies can be administrated orally as passive therapy for dogs against intestinal pathogens (Nguyen., 2006, Lizer., 2008). To protect IgY antibody from stomach gastric juice (pH-6), IgY can be encapsulated. Also, combination of sugar with IgY makes it stable at various pH, and temperature when compared with encapsulation preparation cost. Passive immunotherapy needs bulk amount of antibody production, and chicken egg yolk antibody have reliable results to prove it as an applicable technology mainly considering cost effectiveness and efficacy. Competitive exclusion of pathogens is one of the most important beneficial health claims of probiotic usage. Spray dried egg yolk powder as a whole blended with probiotics microbes (*Lactobacillus acidophilus, Enterococcus faecium).* The use of both IgY with probiotics by means of oral administration might give an effective passive therapy to treat gastroenteritis in dogs.

VII.SUMMARY

A report from veterinarians says that *Canine parvovirus* (CPV), *Canine corona virus* (CCV) rates as first opportunistic pathogens causing hemorrhagic gastroenteritis in susceptible dogs and puppies. They are accompanied by secondary bacterial infection in intestine leading inability to absorb nutrients, causing acute diarrhea. The intestinal wall becomes so damaged that bleeding occurs, due to viral and bacterial co-infections. Therefore, puppies die from severe dehydration and shock. These pathogenic virus (CPV) have high morbidity (100%) and frequent mortality up to 10% in adult dogs and 91% in pups. *Salmonella typhimurium were* predominant in causing gastroenteritis in dogs when compared to other bacterial agents. Specific antiviral treatments are lacking and veterinarians are left only to treat the symptoms of disease.

White leghorn chickens were immunized with CPV, CCV, *and Salmonella typhimurium* and their antibody titer was increased by periodic boosters. Eggs collected and egg yolks were separated and purified. The concentration of total protein increased in egg yolk with subsequent booster dosage yields an average of about 57.4 mg/ml and total IgY antibody was 42.3 mg/ml and it remains constant till 150th day of study. Agglutination technique showed the qualitative determination of IgY antibody, Micro agglutination test gave the quantitative raise of IgY antibodies up to titer 1:1280 against *Salmonella typhimurium*. Antigen specific IgY were determined by ELISA showed the highest titre up 1:10000 from 60th day of immunization. DEAE cellulose column purified IgY samples were analyzed for their purity by SDS PAGE. And it showed higher molecular weight protein (180 K Da) of IgY antibody.

Spray dried whole yolk powder was collected from 50th day post immunization of all the 3 antigens and were blended with commercially available probiotics *Lactobacillus acidophilus, Enterococcus faecium.* Passive immunization by means of oral administration of both probiotics with IgY antibodies may be useful in the treatment of dogs with gastroenteritis.

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