World poultry industry is expanding, as the population is increasing. In India, poultry is one of the fastest growing segments of the agricultural sector. The growth rate of Indian poultry sector has been rising at 8 to 10% per annum. As a result, the country is now the world’s fifth largest egg and broiler producer (India Live, 2011). India, the world’s second largest developing country, is contributing to the expansion through the rapid growth of its poultry sector. Farmers in India have moved from rearing country birds in the past to rearing hybrids, which ensure faster growth of chicks, low mortality rates, excellent feed conversion and sustainable profits to the poultry farmers. India also leads in producing specialized products like Specific Pathogen Free (SPF) eggs in Asia, which act as key inputs for manufacturing poultry vaccines and health products (ICRA Rating Feature, 2011).

Key performance metrics for a poultry farm include – feed conversion ratio (FCR), i.e., amount of feed consumed to gain per unit weight, time taken to attain desired weight (for broiler), rate of laying eggs (for a layer), mortality rate and disease resistance. Existing FCR in domestic industry is around 1.8-2.0 (i.e. every 2 kg of feed consumed results in 1 kg weight gain) though companies strive to reduce the same through advances in veterinary science and improved feed mix. Another important aspect is to increase resistance against various diseases to reduce mortality rates. Typical mortality rates in Indian farms are 8-10% while developed countries have less than 5% mortality rates providing potential for further improvement in disease management practices (ICRA Rating Feature, 2011). The diseases in poultry birds not only kill birds but also cause poor growth, poor hatchability and poor egg production. Among the many poultry diseases, enteric diseases are one of the most important groups that affect poultry and are continuing to cause high economic losses. Several infectious agents like viruses, bacteria, fungus and parasites are involved in intestinal disorders (Hafez, 2001).
At early days of age, the main disease problems are *Salmonella* and *E.coli* (Hafez, 2011). With the great expansion of poultry industry, the wide spread occurrence of *Salmonellosis* is considered as one of the most important bacterial diseases of poultry, even the survivor of *Salmonella* infection becomes the carrier for life and become source of infection for other birds. This problem is major concern for breeders as they produce hatching eggs which carries these bacteria and resulting chicks are born with the infection and frequently die (Bharti, 2008). Hence, the *Salmonella* infection is a problem of economic concern to all phases of poultry industry and also a source of food borne transmission of disease to humans. There are mainly two types of non-motile avian *Salmonella* sp. namely *S. gallinarum* and *S. pullorum* that cause fowl typhoid and pullorum disease respectively. Morbidity and mortality due to pullorum disease varies from 0% to 100% whereas, that of 10% to 93% due to fowl typhoid in chicks (Shivaprasad, 2003). *Salmonella* can cause a spectrum of pathological conditions such as acute gastroenteritis and bacteraemia in humans (Chalghoumi et al., 2009). Two serovars, *S. enteritidis* and *S. typhimurium* account for most Salmonellosis associated with foods of animal origin (EFSA, 2007). Therefore, preventive and curative strategies are of great importance to reduce the *Salmonella* colonization in chickens at farm level.

In a common way the diseases are being controlled in the poultry industry by administering the antibiotics. Currently, the antibiotic usage is under scrutiny because there have been increasing percentage of antibiotic-resistance. The recognition of the dangers of antibiotic resistance prompted the ban on sub-therapeutic usage of antibiotics in many developed countries and developing countries are seriously considering a similar ban. Emergence of antibiotic resistance and the need to treat diseases caused by pathogens that do not respond to antibiotics, have put tremendous pressure to look for viable alternatives (Karlsson et al., 2004).
Many products such as organic and inorganic acids (Kim et al., 2005), prebiotics (Flickinger et al., 2003), probiotics (Taras et al., 2007), herbal extracts (Windisch et al., 2008) and antibodies (Cook, 2004) have been evaluated as potential alternative to antibiotics. Among these, passive immunization by oral administration of antibody is a highly attractive and effective alternative approach to control Salmonella in poultry due to its specificity. Oral administration with antibodies derived from mammalian serum, colostrum and monoclonal antibodies (Kuhlman et al., 1988). However, it is very expensive to obtain large quantity of antibody required to prevent or treat diseases.

Chicken egg yolk antibodies (IgY) has considerable attention for preventing and controlling disease as it holds a large number of advantages compared with mammalian IgG including cost-effectiveness, convenience and high yield (Carlander et al., 2000). Under natural conditions, the serum IgY of laying hens is being transferred in large quantities in the egg yolk in order to protect the developing embryo from potential pathogens (Janson et al., 1995). Thus, it is achievable to immunize the hen against specific pathogens thereby allowing the production of IgY with activity against these specific disease causing pathogens. Oral administration of specific IgY has been reported to be effective against a variety of intestinal pathogens (Kovacs-Nolan, 2012). With this background information the present investigation has been carried out to raise chicken egg yolk antibodies (IgY) against Salmonella pullorum, S. typhimurium and S. enteritidis and their characterization for in-vitro and in-vivo efficacy.

Standard strains of S. typhimurium and S. enteritidis were obtained from culture collection centre (MTCC and ATCC). S. pullorum plain antigen was procured from IVRI, Izatnagar. The strains were characterized morphologically and biochemically in the laboratory and it was found that the procured strains and antigen were pure.
Salmonella whole cell antigens were prepared as per the method prescribed by Lee et al., (2002). During antigen preparation the purity, sterility and specificity of antigens were verified by the standard methods. It was found that all the prepared antigens were pure and sterile with their corresponding specificity.

5.1. Generation and characterization of Anti-Salmonella-IgY in white leghorn chickens: White leghorn chickens were immunized with prepared Salmonella whole cell antigen (1x10^9 cells/kg of body weight) in pectoral muscle. Then the chickens received booster doses with the same concentration at 14 days interval. The eggs were collected and stored at 4°C. It was essential to have the proper egg production, if the layer chickens were reared with the aim of antibody generation, because the eggs laid by immunized chickens were the main source of antibodies. There were many possible factors which may influence the egg production in the regular layer farms, but during antibody generation there was one more possible factor which was suspected to affect the laying capacity of chickens, that was immunization with the prepared antigens. Hence, the egg laying performance of immunized chickens were monitored for 20 weeks from the date of first immunization. It was found that the immunization with Salmonella whole cell antigen did not influence egg production and the egg laying capacity of chickens were influenced by factors other than immunization. Similar observation with other antigens was reported by Schade et al., (1994).

During antibody purification from immunized eggs, some physical parameters of eggs such as egg weight, volume of yolk and albumin, weight of yolk and albumin and egg shell weight were studied in the weekly intervals in order to observe the variations in the IgY concentration due to stress, immunization and age of the birds. The volume of yolk was found to be 6-7ml in the eggs laid by 19 weeks old chicken. Then it was gradually increased and reached the stable volume around 15ml in the 24th week of chicken age. Similar age
dependency was observed for other parameters. But, yolk volume was taken for the consideration since it was the source of IgY. It was quite interesting observation that the concentration of IgY was directly proportional to volume of yolk. The volume of yolk was dependent on the size of egg; the size of egg was dependent on the age of chicken (Fig.10a-d). On the whole the development of total IgY in egg yolk of immunized hens was dependent of age. This finding was similar to the previous observation made by Pauly et al., (2011).

The antibodies were purified from the egg yolk by Polyethylene glycol (PEG) extraction method prescribed by Polson et al., (1980). The IgY-extracts were further purified by DEAE cellulose ion exchange column chromatography. IgY antibodies were also purified by water dilution method developed by Akita and Nakai (1992). PEG extraction method was adopted for the entire study because the final volume of the extract was only around 2ml; the smaller volume obtained was found to be an advantage to store the IgY-extract for further studies. But in the case of water dilution method the final volume of IgY extract was huge and further concentration technique was necessary to concentrate the IgY before storage. The concentration of protein in the IgY-extracts was estimated with the extinction coefficient of 1.33 for IgY (Pauly et al., 2011). As discussed earlier the total IgY concentration was dependent on age of the bird, size of the egg and volume of yolk (Li et al., 1998). Like protein concentration the total IgY concentration was also found to be low i.e., 12.57 ± 0.57 mg/ml during the 19th week of chicken age, then it was found to be relatively constant during the study period of 20 weeks.
increased and attained the concentration of $30.24 \pm 0.25$ mg/ml after 25th week of birds age. The total IgY concentration of each IgY-extracts were 29.93mg/ml, 30.56mg/ml and 30.24mg/ml for Anti-\textit{S.pullorum}-IgY, Anti-\textit{S.tyhpimurium}-IgY and Anti-\textit{S.enteritidis}-IgY respectively. This result revealed that there was no considerable variation in the total IgY concentration among the chickens immunized with different \textit{Salmonella} antigens, which indicated the total IgY concentration was independent of the type of antigens used to generate antibodies as reported by Lee \textit{et al.}, (2002). The purity of IgY-extracts was determined by SDS-PAGE technique (Laemmli, 1970). A clear 180 kDa protein bands were observed in the all the lanes. It was compared with the protein band obtained in the lane loaded with commercial standard IgY (Genei, Bangalore). Bands observed in test lanes and standard IgY lane were similar, which indicated the purity and also confirmed the molecular weight of the IgY extracted from the egg yolk. Some other minor impurities protein bands were also observed, but it was negotiable.

Specific reactivity of Anti-\textit{Salmonella}-IgY in serum and IgY-extracts with their corresponding antigens was determined by Rapid slide agglutination test. Agglutination reaction was observed within 2minutes after mixing the serum and antibody solutions with respective antigens separately. The observation was compared with the agglutination reaction of standard anti-serum (IVRI, Izatnagar) with the \textit{Salmonella} antigens, which was considered as positive control for interpretation of test results. The result indicated the presence of Anti-\textit{Salmonella pullorum}-IgY, Anti-\textit{S. tyhpimurium}-IgY and Anti-\textit{S. enteritidis}-IgY in the serum samples and egg yolks. With this qualitative determination further titration of specific IgY was carried out by indirect ELISA and Micro-Agglutination Test (MAT). The specific antibody level in chicken serum and egg yolk was estimated by ELISA. The level of specific antibodies against the respective antigen in serum was increased after 1 week and slowly it reached the maximum titer on day 21st from the date of initial immunization.
However, the specific antibody level in the egg yolk was very weak on 21st day and gradually increased and reached the peak on 35th and 49th day. The titer of specific antibody was found to be 1:100000 on 35th Day and the titer were maintained with booster doses. Results indicated that there was a delay in the appearance of Anti-Salmonella-IgY in yolk when compared to serum after the first immunization. It was possibly due to the gradual accumulation of IgY during the yolk formation period by selective active transport (Kitaguchi et al., 2008) similar findings was reported by Mahdavi, et al., (2010). The level of specific antibody against the antigen of interest was also determined by Micro-Agglutination test (MAT) as per the procedure described by Nurhadi et al., (2003) with slight modification. The titer was defined as the highest dilution in which the agglutination reaction was observed. In MAT analysis, the specific IgY in the egg yolk was detectable on 14th day after initial immunization and the peak titer was observed up to 1:2560 dilutions on day 49th. Micro-Agglutination test is economical in its use of antibody and is fairly sensitive. The levels of specific antibodies against Salmonella whole cell antigens were determined in both the IgY-extracts of Immunized yolk and un-immunized yolk by ELISA in order to determine the specific IgY ratio between Immunized eggs and un-immunized eggs. The specific antibody titer in the immunized egg yolk was significantly higher than the un-immunized egg yolk. This indicated that the production of specific IgY could be efficiently elicited in chickens using simple protocols of immunization and extraction, this finding was comparable with the report of Guimaraes et al., (2009).

5.2. Physicochemical properties and stability of IgY: In order to evaluate the efficacy of IgY in the prevention and treatment of Salmonella infection in chickens, the stability of IgY was investigated at different physicochemical conditions. The specific reactivity of IgY when incubated as purified IgY or as liquid yolk at different temperature, different pH, pepsin and Trypsin treatment effect were
assessed by measuring the residual activity using ELISA after each treatment. The results showed that the purified IgY was stable at 4°C, 10°C, 25°C and 37°C. Approximately 25% of its activity was lost at 60°C and then significantly decreased at 70°C. It was almost completely lost at 80°C. The purified IgY was stable between pH 4.0 and pH 10.0, it has retained only 20% of its activity at pH 2 and completely lost its activity at pH 12.0. In liquid yolk, IgY was relatively stable to high temperature at 80°C and complete loss of activity at 90°C. The pH stability of IgY with liquid yolk was found to be from pH 3.0 to 11. The activity of purified IgY after pepsin treatment was almost completely lost but 30% of IgY activity was recovered when it was in liquid yolk. In contrast to pepsin treatment, purified IgY showed broad stability to trypsin, approximately 80% of the antibody activity was retained even after 4 hours. Trypsin did not seem to have any detrimental effect on IgY when it was in liquid yolk. These results revealed that IgY was relatively stable to high temperature and broad pH range in the presence of its natural form (egg yolk) and IgY was more resistant to the effects of trypsin compared to pepsin. These observations were indicating that once the IgY passes the acidity of the stomach, it could retain most of its activity and therefore, can combat or minimize the effect of intestinal pathogens such as *Salmonella* when it is given as natural form (egg yolk). Similar results were reported by Jaradat and Marquardt (2000).

### 5.3. In-vitro efficacy of Anti-\textit{Salmonella}-IgY:

Growth inhibition assay was performed to investigate that the binding activity of Anti-\textit{Salmonella}-IgY could inhibit the *Salmonella* growth in the liquid medium. The growth of *S. typhimurium* and *S. enteritidis* was similar in patterns that showed a lag phase of 0 to 2 hours, exponential phase of 2 to 6 hours and then stationary phase after 6 hours of incubation at standard conditions. With the similar conditions, the growth of *S. typhimurium* and *S. enteritidis* with their respective specific IgY and non-specific IgY was plotted for growth inhibitory assay. In this assay the IgY concentration i.e., 360mg/ml was used on the basis of
previous study done by Lee et al., (2002). The growth of *S. typhimurium* and *S. enteritidis* with specific IgY showed significant reduction after 4 hours of incubation when compared to the growth with non-specific IgY. The results were comparable to the report of Lee, *et al.*, (2002). As a result it was observed that both the *Salmonella*-specific IgY were found to inhibit the growth of homologous cells in a liquid medium. The mechanism of action by which the antibodies suppress the growth was not clearly understood, but there are some proposed reasons discussed by Lee *et al.*, (2002); Sunwoo *et al.*, (2010) and Mahdavi *et al.*, (2010). The binding of specific IgY to bacterial surface components could cause some structural alterations of the bacterial surface, which may block the opportunity to take nutrients and proliferate. This could be because antibodies were generated against whole bacterial cell, which could possess binding activities against various epitopes of the bacterial surface as is the characteristics of a polyclonal antibody. Therefore, binding activities of IgY against bacterial surface components, including fimbriae and outer membrane protein may cause the growth inhibition. The results revealed that the specific binding activity of IgY is an important factor for the major antibacterial property. However, it is essential to conduct more intensive studies to explore the exact mechanism of inhibition.

### 5.4. Immune Egg yolk Powder for In-vivo efficacy study:

The present investigation was previously aimed to compare the efficacy of purified IgY and Immune egg yolk powder separately. On the basis of review of literature and observations made during the study period, it was found that the usage of purified IgY has its own limitations such as method of purification (to be less time consuming and low cost with maximum yield), mode of administration and ability to withstand in the gastrointestinal tract of chicks. Therefore, some alternative methods were sought for the safe delivery of IgY to the intestinal tract. For this reason, the micro-encapsulation of IgY was attempted by the sodium alginate method with the aim to supplement the broiler feed in
the form of Freeze dried IgY-microcapsule powder. Though the encapsulation process was successful, the spherical structure of microcapsule got damaged upon freeze drying. Furthermore, it was confirmed in some recent studies by other researchers and also in present investigation that the stability of IgY to different physicochemical conditions was relatively higher if it is in natural form (egg yolk) when compared to purified form. In addition to this, Micro-encapsulation method is not economically suitable for animal feed supplementation. Hence, the current study has been constrained to prepare immune egg yolk powder and its characterization to use it as broiler feed supplement.

The yolk were separated from the hyper-immune eggs and diluted with sterile distilled water. Then the diluted yolk mixture was dried by two methods namely freeze drying and spray drying at two different range of inlet and outlet temperature. Afterwards, both Freeze dried yolk powder (FYP) and Spray dried yolk powder (SYP) were characterized. IgY was purified from the yolk powders and the specific reactivity was primarily confirmed by Rapid slide agglutination method and then the titer of specific IgY was found to be 1:100000. The intact structural stability of IgY was confirmed by Native-PAGE analysis in which 180 kDa protein band was observed in all the preparation. It was found that there was no considerable difference in the stability and reactivity of IgY between FYP and SYP. But, the moisture level was drastically higher in FYP when compared to SYP. Even, the time taken to prepare yolk powder by freeze drying method was remarkably higher than spray drying process. Therefore the spray drying method was found to be a feasible one for yolk powder preparation with the consideration of rapidity, low moisture level and low cost along with adequate stability of IgY up on spray drying temperature. The results revealed that the optimum temperature for spray drying of Egg yolk was found to be 100°C inlet and 80°C outlet or 80°C inlet and 60°C outlet since they resulted in negotiable IgY degradation. With the aim to increase the stability of IgY during spray
drying, mannitol was used as protectant since it was widely used in food products to improve texture, resist moisture and prevent foods from browning effects. Similar attempt was reported by Gujral et al., (2012). The present study result revealed that, the activity of IgY in spray dried yolk powder with and without mannitol was found to have no significant variation. But, the moisture content was considerably reduced when mannitol was used. The amorphous condition was high when the yolk alone was dried, but the powder was less amorphous when the yolk was dried with mannitol. Therefore the mannitol could be used for yolk powder preparation to reduce the moisture level and enhance the texture of the powder, which could help for a long term storage.

5.5. In-vivo efficacy of Anti-Salmonella-IgY against experimental Salmonellosis in Broiler Chicks: Though the study was carried out to generate and characterize chicken egg yolk antibodies against S. pullorum, S. typhimurium and S. enteritidis, the in-vivo efficacy of Anti-Salmonella-IgY against experimental Salmonellosis was performed only for Salmonella enteritidis (SE) with the ethical concern to use the number of animals as minimum as possible for experimental purpose. S. enteritidis and S. typhimurium are capable of causing highly virulent systemic disease in young chicks at early days of age, but in older birds they lead to persistent colonization of the gastrointestinal tract without causing discernible symptoms or illness (Holt et al., 1999 and Beal et al., 2004). In the present study also, the similar observation has been made, that all the birds that had received oral SE infection were dull and had diarrhea in early days of age, then it disappeared. During the experimental period, no clinical symptoms of infection and mortality were observed.

Estimation of SE colonies in the Caecal content was carried out by culture technique. Negative control group showed the highest number of colonies in all euthanasia when compared to the treated groups. Within the group the number of SE colonies was high till 21st
day. Then it was gradually decreased and very less number of colonies were obtained on 42\textsuperscript{nd} day euthanasia. This indicated that the bird’s immune response alone was not able to combat pathogen in the early days of age; after 21\textsuperscript{st} day it has grown enough to combat with the pathogen but failed to eliminate the pathogen from the system completely. It was observed that there was a significant difference in number of SE colonies in the Caecal content between treated and untreated groups. Similar observations were made in ducklings (Fulton \textit{et al.}, 2002), piglets and calves (Yokoyama, 1998). Organ invasion of SE was studied by detecting SE in liver and spleen tissue samples on experimental days 21\textsuperscript{st}, 28\textsuperscript{th} and 42\textsuperscript{nd} day. The SE infection was detected in organ tissues of many experimental groups on 21\textsuperscript{st} day. However, as the time elapsed from the initial day of oral infection fewer and fewer positive samples from these organs were found on 28\textsuperscript{th} day. All experimental groups showed no positive case on 42\textsuperscript{nd} day.

When comparing the culture result of Caecal content and the organ invasion result, it was understandable that most of the treated group has showed negative result for SE detection in the organ samples on 28\textsuperscript{th} day except the groups treated with 0.05% immune egg yolk powder and 7.5% un-immunized yolk powder, it was likely due to the SE colonization in the intestinal tract itself was being prevented maximally by the treatment given immediately after the experimental infection and later on. Hence there was no subsequent invasion of SE in the organs after 21\textsuperscript{st} day onwards. In addition to this, broilers were able to clear systemic infection by natural immune response, but the intestinal carriers remain in the untreated group till 42\textsuperscript{nd} day. This intestinal carrier status is most important in horizontal transfer of the infection to the other normal flocks of close proximity by cross contamination, if the birds are untreated in the regular poultry practice. Contamination by faecal shedding during transportation and processing of broilers is also possible (Gurtler, 2004). These results suggested that the specific antibodies could affect
the degree of colonization; this was supported by the investigation of Tusborkura et al., (1997) which indicated that the antibody used did not possess any bactericidal or bacteriostatic effect in-vitro, but led to altered growth characteristics with clumping of the bacteria. It is therefore possibly inferred that one of the major mechanisms of action could be blocking of bacteria to adhere to the intestine.

In the first part of the in-vivo efficacy study, the capability of Anti-Salmonella-IgY to control or prevent the colonization of Salmonella in the gastrointestinal tract was evaluated. Here, it was aimed to observe the effect of feed supplementation with immune egg powder on growth performance of challenged broiler chickens linked to Salmonella infection. It was rarely stated in the scientific literature about the negative effect of experimental Salmonellosis by S. enteritidis and S. typhimurium on broiler growth performance (Hegazy and Adachi, 2000; Nakamura et al., 2002). In the present investigation, it was found that the experimental Salmonellosis has lead to growth performance deterioration i.e., decreased body weight (BW) gain. Growth performance study of experimental broiler chickens showed 21.77% of decreased BW gain and FCR increased from 1.86 (mean FCR of specific IgY treated group) to 2.02 in the challenged-untreated group (Negative control) when compared to the groups treated with different concentration of immune egg yolk powder. Similar observation for growth performance deterioration in the Salmonella challenged groups were also reported by Chalghoumi et al., (2009).

Prevention of Salmonella colonization was also observed in the group treated with 7.5% un-immunized yolk powder. These results were in accordance with Kasssaify and Mine, (2004) report which revealed, that it wasn’t clear how non-immunized egg yolk powder was able to prevent Salmonella colonization or organ invasion in Broiler chicken. The precise mechanism by which the egg yolk component prevents or eliminates infection is still under investigation. However,
the present study results clearly showed that the colonization of *Salmonella* has been prevented significantly even in very low concentrations of Immune egg yolk powder than in 7.5% un-immunized yolk powder. It was attributed by the presence of Anti-*Salmonella*-IgY in the immune egg yolk powder but not in un-immunized yolk powder. It was also observed that there was 8-10% of increased BW gain in the groups treated with immune egg yolk powder than in Normal group and Positive control group (treated with antibiotic); it could be due to the nutritive value of egg yolk components, which could have helped the broiler chickens to gain weight while Anti-*Salmonella*-IgY combat pathogens and prevented their colonization in the gastrointestinal tract.

In conclusion, the binding activity of Anti-*Salmonella*-IgY has resulted in inhibiting bacterial growth in the liquid medium (*in-vitro*). The oral administration of Chicken egg-derived antibodies were found to be effective in preventing the early *Salmonella* colonization in the intestinal tract of broiler chickens (*in-vivo*) and has helped them to gain weight as functional feed additive. It has been indicated that inclusion of Hyper immune egg yolk powder in poultry feed may be a useful tool in controlling *Salmonella* infection. However, more intensive studies are needed for the successful application of egg derived antibodies as a tool to control infection in poultry and other livestock. The present study may help to some extent for the justification of applications of IgY antibodies as prophylactic and therapeutic agent. The study could form a platform for further research on egg yolk antibodies and its commercial application in India.