3. MATERIAL AND METHODS

3.1. Materials

3.1.1. Samples:

From four broiler chicken flocks at Damietta governorate, a total of 1073 samples were collected from 310 chicken (142 of apparently healthy broiler chicken, 43 of diseased and 125 of dead broiler chicken showing whitish watery diarrhea) (Table 1). Total number of flocks, morbidity, mortality, type and numbers of samples were illustrated in Table (2), typing and numbering of samples according to the status of chicken was cleared in Table (3).

Table (1): Number of chicken collected for sampling

Flock	Total number of birds / flock	Healthy chicken	diseased chicken	Dead chicken	Total
Flock 1	4899	39	8	37	84
Flock 2	7279	35	20	33	88
Flock 3	5929	31	7	18	56
Flock 4	6873	37	8	37	82
Total	24.980	142	43	125	310

Table (2): Flocks, morbidity, mortality, type and number of samples collected

flocks	Visits	No. of birds / flock	Age / day	Morbidity%	Mortality%	Type and number of samples collected				
						Yolk sac.	Cloacal swab	Gall bladder	Spleen	liver
Flock1	1st visit	4899	2days	2.04	1.02	28	28	28	28	28
	2nd visit		10days	1.46	0.93	-	23	23	23	23
	3rd visit		20days	1.02	0.69	-	15	15	15	15
	4th visit		40days	0.71	0.10	-	18	5	5	5
Total No.						28	84	71	71	71
Flock 2	1st visit	7279	2days	2.08	1.73	16	16	16	16	16
	2nd visit		10days	1.64	1.36	-	22	14	14	14
	3rd visit		20days	1.09	1.05	-	33	22	22	22
	4th visit		40days	0.75	0.41	-	17	11	11	11
Total No.						16	88	63	63	63
Flock 3	1st visit	5929	2days	1.52	0.67	18	18	9	9	9
	2nd visit		10days	0.86	0.43	-	11	10	10	10
	3rd visit		20days	0.50	0.35	-	17	13	13	13
	4th visit		40days	0.37	0.25	-	10	6	6	6
Total No.						18	56	38	38	38
Flock 4	1st visit	6873	2days	2.27	1.40	17	23	17	17	17
	2nd visit		10days	1.45	0.96	-	22	15	15	15
	3rd visit		20days	0.77	0.32	-	21	14	14	14
	4th visit		40days	0.58	0.13	-	16	10	10	10
Total No.						17	82	56	56	56

% Of morbidity and mortality were calculated according t the total no. of the flock.

Table (3) : Samples according to the status of birds.

Flock	Total No. of birds / flock	Type of samples	No. of collected samples			Total
			Healthy	diseased	Dead	
Flock 1		Cloacal swabs	39	8	37	84
		Gall blader	30	4	37	71
		Yolk sac	13	5	10	28
		Spleen	30	4	37	71
		Liver	30	4	37	71
Total			142	25	158	325
Flock 2		Cloacal swabs	35	20	33	88
		Gall bladder	13	17	33	63
		Yolk	8	0	8	16
		Spleen	13	17	33	63
		Liver	13	17	33	63
Total			82	71	140	293
Flock 3		Cloacal swabs	31	7	18	56
		Gall bladder	15	5	18	38
		Yolk sac	9	2	7	18
		Spleen	15	5	18	38
		Liver	15	5	18	38
Total			85	24	79	188
Flock 4		Cloacal swabs	37	8	37	82
		Gall bladder	21	8	27	56
		Yolk sac	5	5	7	17
		Spleen	21	8	27	56
		Liver	21	8	27	56
Total			105	37	125	267
Total of all samples			**414**	**157**	**502**	**1073**

3.1.2. Media used:

3.1.2.1. Buffered peptone water: (Oxoid)

It was used as pre-enriched media.

3.1.2.2. Rappaport vassiliadis: (RV) (Oxoid)

It was used as selective enriched media

3.1.2.3. Salmonella Shigella agar: (S.S) (Oxoid)

It was used as a selective media for isolation of ***Salmonellae***.

3.1.2.4. Tetrathionate broth base: (TB) (Oxoid)

It was used as selective enriched media. The complete medium (with added iodine) should be used the same day as it is prepared.

3.1.2.5. Tryptic soya agar: (TSA) (Oxoid)

It was used as a preservative media

3.1.2.6. Xylose lactose desoxycholate medium: (XLD) (oxoid)

It was used as a selective medium for isolation of ***Salmonellae***.

3.1.2.7. Brain heart infusion agar: (Oxoid)

It was used for preparation of ***S.* Enteritidis** antigen.

3.1.2.8.Brain heart infusion broth: (Oxoid)

It was used for preparation of ***S.* Enteritidis** antigen.

3.1.2.9.Sabouraud dextrose agar: (Oxoid)

3.1.3. Media used for biochemical identification of *Salmonellae*:

3.1.3.1. Christensin's urease agar slants (Oxoid):

It was used for detection of urease enzyme activity of the isolates.

3.1.3.2. Glucose phosphate broth (Oxoid):

It was used in Methyl red reaction and Voges Proskauer test.

3.1.3.3 Peptone water 2% (Oxoid):

It was used for detection of indole production

3.1.3.4. Triple sugar iron agar medium (TSI) (Oxoid):

It was used for detection of hydrogen sulphide as well as detection of sugars (glucose, lactose and sucrose) fermentation and gas production.

3.1.3.5. Semisolid nutrient agar:

It was used for motility test and for storage of isolated strains

3.1.3.6. Simmon's citrate agar (Oxiod):

It was used for citrate utilization test.

3.1.3.7. Suger fermentation media:

1% peptone water containing 1% of the following sugars: glucose, maltose, mannitol, dulcitol, rhamnose, sorbitol and trehalose with 1% Andrade‘s indicator.

3.1.4. Chemicals and reagents used for biochemical identification of *Salmonellae* (Cruickshank *et al*., 1975):

3.1.4.1. Hydrogen peroxiode solution3%:

It was used for catalase test.

3.1.4.2. Kovac's reagent:

It was used for the indole test**.**

3.1.4.3. Methyl red solution (0.04%):

It was used for methyl red test

3.1.4.4. 40% Potassium hydroxide solution and 5% solution of alpha Naphthyol in absolute ethanol:

It was used for the Voges-Proskauer test.

3.1.4.5. Andrade‘s indicator:

It was used for sugar fermentation test.

3.1.4.6. Tetramethyl phenylene diamine dihydrochloride solution 1%:

It was used for oxidase production test.

3.1.5. Stain used:

3.1.5.1. Gram's stain (Quinn *et al*., 1994)

It was used for staining of microorganisms.

3.1.5.2. Safranin solution :

0.5 g Safranin (Mukkienbery) in 100 ml distilled water. It was used for staining of ***Salmonella*** antigen in microagglutination test (MAT).

3.1.6. Diagnostic *Salmonella* antisera:

3.1.6.1. polyvalent O and H *Salmonella* antisera.

3.1.6.2. Monovalent *Salmonella* antisera (Difco).

3.1.7. Material used for experiments:

3.1.7.1. Experimental chickens:

A total of three hundred and ten, day-old Hubbard broiler chicks of mixed sex that obtained from Cairo Poultry Company. In 10th Ramadan city were used for evaluation of the protective value of the prepared ***S. Enteritidis*** bacterin in comparison with a probiotic against ***S. Enteritidis*** challenge. The chicks were taken from a breeder flock free from salmonellosis. The birds were kept under complete observation for six weeks (experimental period) in separate thoroughly cleaned and disinfected houses and provided with feed and water *adilbitum*.

3.1.7.2. The ration:

Commercial starter and grower broiler chicken ration were given till 21 and 35 days of age, respectively, while finisher ration was used till 42 days of age (end of the experiment). The used commercial balanced ration based on yellow corn or soyabean that met the **National Research Council (NRC) (1984)** broiler chicken requirements. The starter ration contained crude protein-not less than 21%, crude fat-not less than 2.94%, crude fibers-not less than 2.35%, metabolizing energy-not less than 3054 Kcal/kg ration and used for the first 4 weeks. The finisher ration contained crude protein-not less than 17.15%, crude fat-not less than 2.5%, metabolizing energy-not less than 3020 Kcal/kg ration and used for the remaining of the experimental period. The ration was containing no growth promoters but contained coccidiostate.

3.1.7.3. Vaccines:

3.1.7.3.1 Hitchner B_1: ceva Sant animal, Batch No. 0505151A.

3.1.7.3.2. H 120: Intervet company.

3.1.7.3.3. Reasortant AI virus vaccine: Inactivated (H_5N_1 subtype, Re 1 strain). Produce by Harbin Weike Biotechnology development Co. Batch No. 2009017.

3.1.7.3.4. IBD vaccine (Gumboro 228 E): Intervet intermediate. Batch No.08824EJ01.

3.1.7.3.5. La Sota Strain: Intervet company.

3.1.7.4. Experimental vaccine:

Locally prepared inactivated ***S.* Enteritidis** vaccine (Inactivated oil emulsion bacterin containing ***S.* Enteritidis).**

3.1.7.5. Probiotic used:

The probiotic that used in the experiment was a commercial preparation containing (***Lactobacillus* acidophilus**, ***Enterococcus* faecium**, ***Lactobacillus* plantarum**, ***Lactobacillus* casei**) Plus Potassium, Vitamin A, D3, E and K,

Riboflavin, Pantothenic Acid, Thiamine, Niacinamide. Manufactured by Bomac Vets Plus, USA. Batch No.,: A704.

3.1.7.6. Strains used for challenge and serological tests:

Strain isolated from chicken at Damietta governorates (***S.* Enterditis**), was used on this experiment for challenge and also for preparation of antigen for ELISA and micro-agglutination serological tests.

3.1.7.7 serum samples:

Sera were collected from wing vein of chickens at different intervals.

3.1.8. Material used for micro- agglutination test:

- Phosphate buffer saline 0.85% (PBS pH 7.2).
- V-shaped microplate was used (Hochest Behring)
- Safranin O-stained microtest antigen.

3.1.9. Materials and reagents used for the Enzyme Linked Immunosorbent Assay (Indirect ELISA). (Kim *et al*., 1991):

3.1.8.1. Solutions and buffers.

3.1.8.1.1 Coating buffer (0.5 M Carbonate buffer, pH 9.5):

Sodium carbonate	0.16 g
Sodium bicarbonate	0.29 g
Distilled water up to	100 ml

It was used for coating ELISA plates and dilution of antigen.

3.1.8.1.2 Phosphate buffer saline (PBH), pH 7.2.:

Potassium chloride	2.9 g
Potassium di-hydrogen phosphate	8.0 g
Di- Sodium hydrogen phosphate	0.2 g
Distilled water up to	1000 ml

3.1.8.1.3. Blocking buffer:

Bovine serum albumin	1 g

PBS pH (7.2) 100ml

3.1.8.1.4. Diluting buffers:

PBS pH (7.2)

3.1.8.1.5. Substrate reagent (Biochek poultry immunoassays):

Substrate Tablets: PNPP (p-Nitrophenyl phosphate) tablets to dissolve with Substrate buffer.

Substrate Buffer: Diethanolamine buffer with enzyme co-factors.

3.1.8.1.6. Washing buffer (Biochek poultry immunoassays):

Powdered phosphate Buffered saline with Tween 20.

3.1.7.1.7. Stopping solution (Biochek poultry immunoassays):

Sodium Hydroxide in Diethanolamine buffer.

3.1.8.2. Biological materials:

3.1.8.2.1. Antigen used for ELISA:

Antigen was prepared from local isolated ***S.* Enteritidis** strain.

3.1.8.2.2. Conjugate reagent (Biochek poultry immunoassays):

Sheep anti-chicken: alkaline phosphatase in Tris buffer with protein stabilizers, inert red dye and sodium azide preservative (0.1% W/V).

3.2. Methods

3.2.1. Sampling:

Samples were collected separately under strict hygienic measures and were transferred in ice box directly to the laboratory for bacteriological examination.

3.2.2. Isolation and identification of *Salmonella* isolates: (OIE, 2004)

- 1 g of each sample was inoculated in 9ml buffered peptone water and incubated over night at 37 C for 18 h as pre-enrichment for ***Salmonellae***.

- 0.1ml of pre-enrichment culture was transferred to RV and incubated for 24 h at 41.5 C (as selective enrichment).
- In parallel 0.1ml of the same pre-enriched culture was transferred to a tube containing 10ml of TB and incubated at 37 C for 24 h (as selective enrichment).
- A loopfull from both enrichment incubated broth (RV and TB) was streaked on both S.S agar and XLD medium and incubated for 24 h at 37 C.
- Red colonies with black center on XLD medium and white colonies with black center on S.S agar were considered as suspected colonies and separate colony was picked up on trypticase soya agar slant (TSB) for further microscopical examination, biochemical and serological identification.

3.2.3. Microscopical examination:

Suspected colonies were stained by Gram's stain according to **Quinn *et al*. (2002**) and examined microscopically for Gram negative short rods colonies.

3.2.4. Biochemical identification:

The purified isolates of ***Salmonellae*** were examined by different biochemical reactions according to **Quinn *et al*. (2002)** either by :

3.2.4.1. Urea hydrolysis.

3.2.4.2. Indole test:

Suspected colonies were tested for indole production.

3.2.4.3. Oxidase test:

3.2.4.4. Triple sugar iron agar:

Suspected colonies were tested for H_2S production on triple sugar iron (TSI).

3.2.4.5. Citrate utilization test.

3.2.4.6. Methyl red test.

3.2.4.7. Voges Proskaur test.

3.2.4.8. Sugar fermentation test.

3.2.4.9. Motility test:

In U-shaped tube contains semisolid nutrient medium, suspected colonies were streaked from one side and incubated for 24 h at 37 C.

3.2.5. Serological identification:

The isolate preliminary identified biochemically as ***Salmonella*** were subject to serological identification according to **Chairman *et al*. (1975)** using slide agglutination test

Suspected ***Salmonellae*** were cultured on trypticase soya agar slants for 24 h at 37 C. A loopfull from the culture was suspended in drop of phosphate buffer saline (pH7.4) on a slide, to make a homogenous suspension, then a drop of ***Sallmonella*** anti-sera (separate "O" and "H" factor) was added to the suspension with standard loop and thoroughly mixed to bring the organisms in close contact with anti-sera. Positive agglutination occurred within a minute and could be easily seen with the naked eye as a form of granules or follicles. A delayed or partial agglutination was considered as negative or false.

Polyvalent "O" and "H" ant-sera were first tried to assure that the suspected isolates were ***Salmonella***. Positive culture were then tested with each of the "O" grouping sera followed by the respective mono-specific "O" and "H" anti-sera factors followed by phase I and phase II in order to determine the complete antigenic formula.

3.2.6. Preparation of local *Salmonella* bacterin:

Bacterin was prepared from ***S.* Enteritidis** field strain according to the technique of **Timms *et al*. (1990)**. ***S.* Enteritidis** isolate from semi-solid media was inoculated into nutrient broth at 37 C for 24 h, then to nutrient agar at 37 C for 24 h, after which was grown on brain heart infusion agar kept at 37 C for 48 h in Roux flask. Growth was harvested in normal saline, inactivated with 1 % formol saline for 24 h, at room temperature. Using MacFerland matching tube, washed concentrates of inactivated bacteria were suspended in saline and adjusted to contain 10^{11}CFU/ ml. Sterility test was applied. Sterile bacterin was obtained by adding equal volume of incomplete ferund's adjuvant to adjusted washed concentrate of inactivated bacteria and kept at refrigerator until used.

3.2.7. Quality control tests of the prepared bacterin:

The prepared ***S.* Enteritidis** bacterin was tested for purity, complete inactivation, sterility and safety according to the Standard International Protocols as described by the **British Veterinary Codes (1970)** as follows:

A. Purity test:

The test was done before formalin inactivation of ***S.* Enteritidis** strain. It was applied to confirm that the broth culture of ***S.* Enteritidis** strain did not contain any contamination by other organisms before inactivation. Such purity was detected by inoculation of the broth culture onto S.S agar and incubated at 37 C for 24 h. Appearance of pure colonies of ***S.* Enteritidis**, pure ***S.* Enteritidis** organism after Gram staining and biochemical characterization of the organism indicated culture purity.

B. Completion of *S*. Enteritidis inactivation:

In assurance that the used ***S*. Enteritidis** organisms were completely inactivated, S.S agar media were inoculated with formalin inactivated bacteria. After 24-48 hours incubation at 37 C, no visible growth of ***S*. Enteritidis** indicated complete inactivation of the organism.

C. Sterility test;

The prepared ***S*. Enteritidis** bacterin was confirmed to be free from any fungal contaminants by inoculation of it onto Sabouraud dextrose agar plates and incubated at 25 C for 7 days. Also the bacterin was inoculated on PPLO broth tubes and agar plates and incubated at 37 C for 72 h and 14 days, respectively in Co_2 incubator to ensure the freedom of the bacterin from mycoplasma organisms.

D. Safety test;

Ten, day old broiler chicks were inoculated subcutaneously with a large dose of the prepared bacterin (ten fold the normal bacterin dose).

The chicks were observed daily for 7 successive days for any signs of local reactions, clinical signs or deaths.

3.2.8. Preparation of the challenge inoculum:

Broth culture of ***S*. Enteritidis** field strain was centrifuged at 3000 r.p.m for 10 min. Sediment was diluted with sterile buffer saline, adjusted using MacFerland matching tube to contain 10^9 CFU/ml. The challenge inoculum was prepared according to the method of **Timms *et al*. (1990)**.

3.2.9. Experimental infection;

At 20 days of age, each bird in the experimentally infected groups was inoculated orally with 0.5 ml/ containing 10^9 CFU/ml ***S*. Enteritidis (Okamura *et al*., 2007)**.

3.2.10. The used probiotic:

It was given in the drinking water at the age of one day and 15 day for 5 days in a dose of 1g/4L water as recommended by manufacturer.

3.2.11. Timing of vaccination:

The prepared whole cell inactivated ***S*. Enteritidis** bacterin was given for the experimental chicks at the first day of age in a dose of 0.2 ml/bird and boostered as a second shot at 10 days of age in a dose 0.5 ml/bird. The bacterin in the two shots was given intramuscularly (I/M).

3.2.12. Experimental design:

Three hundred and ten, day-old Hubbard broiler chicks of mixed sex were used. At arrival, randomly ten chicks were sacrificed and then examined bacteriologically to prove their freedom from ***S*. Enteritidis** infection. A completely randomized design was used as three replicates, each consists of 25 chicks and the groups were divided as the followings;

- Group (1): Blank control negative (non infected-non treated birds).
- Group (2): Control positive (***S*. Enteritidis** infected birds).
- Group (3): (probioic treated and ***S*. Enteritidis** infected birds).
- Group (4): (vaccinated *and* ***S*. Enteritidis** infected birds).

3.2.13. The measured parameters for evaluation of the bacterin efficacy in comparison with the probiotic:

1. Clinical assay:

A. Clinical signs, mortalities and gross lesions:

Birds in the challenged groups were observed daily for three weeks post challenge till the end of the study (6 weeks of age) for the clinical signs or deaths.

Dead birds as well as sacrificed birds were subjected to necropsy for recording the lesions of ***S.* Enteritidis (O'Brien, 1988)**.

B. The performance:

At arrival, the chicks were weighed and then the birds in each group were subjected to weekly determination of the production parameters that include; the body weight BW, the feed conversion ratio (FCR) and the European Production Efficiency Factor (EPEF) according to **Sainsbury (1984)**. These measures were taken till the end of the study (6 weeks of age).

C. Detection of *S*. Enteritidis shedding:

Cloacal swabs were taken from birds in each group just before experimental infection (at 20 days of age) to ensure that the birds free from ***S.* Enteritidis** infection. Weekly after the challenge up to 6 weeks of age, cloacal swabs were collected from each of the infected as well as control group and examined bacteriologically for the presence of ***S.* Enteritidis** organism. Sterile cotton swab was inserted into the cloaca of each bird and rotated gently to collect the clocal sample. Each swab was transferred to 10ml tube of Tetrathionate broth and incubated overnight at 37 C. A loopful from the broth was streaked on S.S agar for ***S.* Enteritidis** isolation. Suspected colonies were identified morphologically and biochemically.

D. Re-isolation of *S*. Enteritidis:

Ten birds from each group post challenge were randomly selected weekly, sacrificed and the liver, intestine, heart and spleen were collected for ***S.* Enteritidis** re-isolation. Samples were inoculated into Tetrathionate broth, incubated at 37 C for 24 h, streaked onto S.S agar and incubated at 37 C for 24 h. Suspected colonies were identified morphologically and biochemically.

2. Immunological assay (detection of the humoral immune response):

Just before the first ***S*. Enteritidis** vaccine dose (at zero hour), a randomly ten identified birds were selected and the blood samples were collected via the brachial vain. Also just before the second booster dose of ***S*. Enteritidis** vaccination (10 days after the first dose), another blood samples were collected as previously from the same birds. Blood samples were collected weekly after the second vaccination till 6 weeks of age . (end of the study). The collected blood samples were allowed to clot overnight at 4 C then centrifuged at 3000xg for 10 minutes. The separated sera were stored at -20 C till used in the serological tests. The antibody titer against ***S*. Enteritidis** was determined using microagglutination test and Enzyme Linked Immuno-Sorbent Assay (ELISA) test.

3.2.14. Safranin O-microtest antigen:

The antigen was prepared according to **Brown *et al*. (1981) and Anon (1971),** for preparation of somatic "O" antigen from ***S*. Enteritidis**, pure cultures of the organisms were inoculated onto slope agar tubes and incubated at 37 C for 24 h, then the bacterial cultures were suspended in a reasonable amount of normal saline and inoculated into Roux containing Thiosulphate glycerin agar. The flasks were then incubated at 37 C for 48 h . Bacterial growth was harvested using 0.5 percent Phenolized normal saline, collected in separate sterile bottles. The culture was killed by boiling for one hour and incubated again at 37 C for 24 h . The suspension was centrifuged for 30 minutes at 4000 r.p.m. The supernatant was decanted, while the sediment was resuspended in phenol saline and centrifuged as described before. The process of washing was repeated three times and the last pellet was resuspended in observable volume of phenol saline and diluted with 0.5 percent phenolized normal saline until it reached a degree of turbidity which corresponded to MacFerland matching tube No. 1 as stock antigen

and refrigerated until used. Safranin solution was added to diluted antigen in one percent and mixed thoroughly by magnetic stirrer and used as required.

3.2.15. Preparation of sonicated *Salmonella* antigen for ELISA:

The ***Salmonella*** antigen was prepared according to the methods of **(Barrow, 1992).** One liter of nutrient broth culture of ***S*. Enteritidis** that had been incubated overnight at 37 C was centrifuged and the pellet was resuspended and washed three times in 10 ml phosphate buffered saline. The suspension was sonicated for 2 minutes at power 6% using a cell disruption (Fisher Model 300) with microtip probe and centrifuged at 5000 xg for 60 minutes. The supernatant was stored at -20 C until used.

3.2.16. Microagglutination test (MAT): (Williams and Whittemore, 1971)

- V-bottom disposable (8 x 12 wells) microtiter plate (Hoechst-Behring) was used. 50 µl (microliter) of 0.85% saline were added in each well (10 wells for each serum sample and one control positive well and one control negative well).
- Thirty µl of saline were added to the first well.
- Twenty µl of serum were added to the first well and mixed with saline (a dilution of 1/5).
- Fifty µl of diluted serum from the first well were transferred to well No.2 which mixed and this gave a dilution of 1/10.
- This was repeated to the 10 wells and the last 50 µl were discarded.
- The well No. 11 was a positive serum control and No.12 was an antigen control.
- Fifty µl of the antigen were added to all the wells (1-10).
- The plates were covered and incubated 24 h at 37 C in the bacteriological incubator.
- Positive agglutination were recorded for those titrations exhibiting definitely

agglutinated cells on the bottom and sides of the V-shaped wells.

- Negative titrations were recorded as those showing no agglutination

-The titer of a particular antiserum was the last dilution to posses definite agglutination

3.2.17. Enzyme–Linked Immunosorbent assaya (indirect ELISA) for detecting *Salmonella esnterica* subsp. *enterica* Serovar Enteriditis antibodies in serum samples: (Kim *et al*., 1991)

1. Coating:

100 µl of 1/20 diluted ***Salmonella*** antigen added to each well of ELISA plate (the 96 Flat bottomed plate). The plates were incubated at 4 C overnight.

The plate contents were decanted and washed with washing buffer (200µ/well) for three times. The solutions or washes are removed by flicking the plate over a sink. The remaining drops are removed by patting the plate on a paper towel.

2. blocking:

100µl of blocking buffer (1% bovine serum albumin in phosphate buffer (pH 7.4))was added to each well of ELISA plate, the plate was covered and the plate was incubated at 37 C for 1/2 hour then decanted, washed three times using the washing buffer and dried as before.

3. Serum dilution:

100 µl of diluting buffer and 100 µl of the tested serum were added to each well to form a dilution of 1/2. Each serum sample was duplicated including the control positive and negative sera as well as the blank control. The plates were covered and incubated at 37 C for 1 h.

The plates were decanted, washed three times using the washing buffer and dried as before.

4. Addition of conjugate:

100 µl of diluted antichiken alkaline phosphatase conjugate (1:1,000 in PBST) was added to each well. The plate was covered and incubated for 2h in humid chamber at room temperature.

The plates were decanted, washed three times using the washing buffer and dried as before.

5. Addition of substrate:

Substrate tablets: PNPP (p-Nitrophenyl phosphate) tablets dissolved with Substrate buffer (Diethanolamine buffer) with enzyme co-factors. 100µl of diluted substrate solution was dispensed per well and left for 5 minutes in dark place.

6. Addition of stopping substrate:

The reaction was stopped by addition of 50 µl /well of Sodium Hydroxide in Diethanolamine buffer.

The absorbance (optical density) of each well was read with a plate reader at 405 nm.