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EVALUATION OF THE IMMUNE RESPONSE TO *SALMONELLA ENTERITIDIS* INFECTION AND VACCINATION USING IMMUNOBLOTTING AND MICRO TITER SERUM PLATE AGGLUTINATION TEST

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ABSTRACT

This experiment was conducted to study the immune response of chickens to S. enteritidis infection or different vaccines either by micro titer serum plate agglutination test or by immunoblot test. Different groups were included: chickens vaccinated by live S. enteritidis vaccine (s/c), chickens vaccinated by locally prepared or commercial S. enteritidis bacterins intramuscularly (i.m), and chickens infected orally or intramuscularly by a pathogenic strain of S. enteritidis. Also, the effect of antibiotic treatment on the serological response in live vaccine or infection groups was studied. Agglutination test could detect 100% positive reactors in the first week post vaccination and challenge. The results revealed that the highest serum micro plate agglutination titer was Log_{10} 3.8 obtained by locally prepared bacterin and also obtained by intramuscular infection with pathogenic S. enteritidis. Immunoblotting could detect reacting sera only in the second week post vaccination or infection. Antibiotic (sulpha-trimethoprime) treatment decreased the number of reacting bands in the immunoblot by 1-3 bands less than those infected and not treated with antibiotics. Intramuscularly infected chicks showed 1-2 bands more than orally infected chicks. Live pathogenic bacteria injected i.m. produced higher geometric mean titers and more bands in the immunoblot than the bacterin prepared from the same strain and injected via the same route.

Key Word: Salmonella Enteritidis, Immune Response Immunoblotting.

INTRODUCTION

The incidence of salmonellosis has been reported by public health authorities throughout the world and, despite intensive eradication efforts, this pathogen is still an important problem for poultry and threatens public health ***(White et al., 1997).***

Production of poultry free from salmonella organisms needs high cost housing, tight control on feed quality, hygiene and management. Biological measures form an integral part of control programmes. This may be done with antibiotics, competitive exclusion, vaccines or combinations of all these ***(Zhang-Barber et al., 1999).***

Over 2400 different salmonella serotypes have been identified. A relatively small number are known to be host-adapted, (*S. gallinarum* and *S. pullorum* in chickens). Salmonella infection is normally via the oral route. The organisms rapidly invade the host through lymphoid tissue, including the Payer's patches and the cecal tonsils in chickens and possibly also the enterocytes of the intestinal mucosa, then, salmonella migrate from the sub-mucosa to lymph nodes and where they are ingested by phagocytic cells. They reach the blood stream, probably intracellular and reside in the spleen, liver and bone marrow ***(Popiel and Turnbull, 1985).***

Some non specific host adapted strains, most commonly *S. typhimurium* and *S. enteritidis* can produce clinical disease in poultry under certain circumstances, such as in young chicks when the cells of the reticuloendothelial system are immature, or during or after stress, chicks develop a systemic infection and salmonella are excreted in the feces.

A number of live vaccines have become available and some new vaccines will appear on the market over the next few years, in addition to Salmonella bacterins which have been used over the past few years with variable efficacy.

Bacterin prepared from *S. enteritidis* resulted in variable reduction in shedding rate and organ colonization ***(Gast et al., 1992; Barbour et al., 1993 and Nakamura et al., 1994).*** On the other hand the use of live attenuated vaccine (9R fowl typhoid vaccine or rough mutant of *S. enteritidis*) conferred strong protection against systemic infection ***(Silva et al., 1981)*** although the vaccine strain retains some virulence, may persist for many months and may be transmitted through the egg ***(Cooper et al., 1994).***

However, the use of salmonella vaccines complicated the distinguishing of vaccinal antibody from natural infection responses by conventional serological screening tests and limited their use ***(Cooper et al., 1994).***

Agglutination tests are the most common techniques for monitoring humoral immune responses ***(Lee et al., 1981, 1983).*** Cellular responses could be detected by delayed type hypersensitivity (DTH) ***(Hassan et al., 1991). Lee et al. (1983)*** found that clearance of salmonella was cell-mediated (CMI) rather than humoral dependant.

Immunoblotting (Western Blot) is one of the methods used in detection of humoral fractional immune response for particular antigens. Nitrocellulose membrane has been successfully used as a solid support for antigens in the detection of their antibodies ***(Borden and Kabat, 1986).***

The aim of this study was to evaluate the immunoblot as a relevant test for solving many questions such as:

a- Do chemotherapeutic agent may alter the immune response to some salmonella antigens?

b- Does the immune response to orally infecting salmonella differ from that given parentrally?

c- What is the difference in the immune response between the live vaccine (s/c) and the killed vaccine?

d- What is the difference in the immune response if a live pathogenic *S. enteritidis* injected intramuscularly (i.m.) and the killed vaccine (bacterin) or the live vaccine?

MATERIAL AND METHODS

A total of 140 one-day-old chicks were divided into 9 groups, 15 chicks / group. Five chicks were sacrificed and 15 random fecal swabs were collected from 9 groups and examined bacteriologically to prove salmonella free status on the first day of age before the experiment was started.

Table (1): Experimental design:

Group	Treatment (vaccination or infection) at 14 days of age	Route	Antibiotic treatment
I	Commercial live vaccine vaccine Salvac® Intervet	s/c	+
II	Commercial live vaccine Salvac® Intervet	s/c	-
III	Commercial bacterin Salenvac vaccine® Intervet	i.m	-
IV	Locally prepared bacterin from *S. enteritidis*	i.m	-
V	Infected with 1ml over night broth culture of *S. enteritidis*	orally	+
VI	Infected with 1ml overnight broth culture of *S. enteritidis*	orally	-
VII	Infected i/m with 1ml overnight broth culture of *S. enteritidis*	i.m	+
VIII	Infected with 1ml overnight broth culture of *S. enteritidis*	i.m	-
IX	Non infected, non vaccinated negative control		-

chemotherapeutic agent (Sulpha – Trimethoprime) given for 3 days 12 hrs post treatment.
All groups were challenged at 4 weeks of age (2 weeks post vaccination with 1ml over night broth culture of *S. enteritidis*).

1- Samples:

Serum samples were collected on the first and 2nd week post-vaccination and then on the first, 2nd and 3rd week post-challenge with *S. enteritidis*.

2- Detection of humoral immune response:

a- Micro titer serum plate agglutination test:

The antigen preparation and procedure were performed according to ***(Cruickshank et al., 1975).*** Reciprocal of the highest dilution of serum that showed clear suspension and distinct agglutination mat at the bottom of the well was the agglutination titer (The initial dilution of serum was 1/5).

b- Immunoblot:

The protocol as described by ***(Kim et al., 1991).*** Briefly, *S. enteritidis* was grown on tryptic soy broth then harvested and treated to prepare the sample for electrophoresis as described by ***(Blackall et al., 1991).*** **T**hen the sample was loaded in gel wells of SDS-PAGE according to Laemmli system ***(Lammeli, 1970).*** Then transfer of protein into nitrocellulose membrane and finally testing the serum of infected chickens and analyzing the results.

3- *S. enteritidis* strain:

An identified pathogenic strain of *S. enteritidis* obtained from Poultry and Fish Diseases Dept., Fac. Vet. Med., Alex. Univ. was used for experimental infection, preparation of a local killed bacterin and preparation of antigen for SDS-PAGE, and micro titer serum plate agglutination test.

4- Locally prepared bacterin:

It was prepared from *S. enteritidis* as described by ***(Timms et al., 1990 and Hegazy 2002).***

6-*S. gallinarium* live vaccine® and *S. enteritidis* bacterin®:

They were kindly provided by Intervet, Egypt, Ltd.

5-Statistical analysis:

The General Linear Model for analysis of variance ***(SAS, 1990).***

RESULTS AND DISCUSSION

Infection of chickens with salmonella involve three stages: first, intestinal colonization where the shedding occur ***(Muir et al., 1998)***, second: invasion beyond gastrointestinal tract can lead to multiplication of the organisms in macrophage-phagocyte system of liver, spleen and other organs ***(Barrow et al., 1987),*** third: extensive bacteriamia which may cause high mortality specially in young birds**.**

Intramuscularly infected chicks showed 1-2 bands more than orally infected chicks.

Comparing group VII (infected i.m., treated with antibiotic) had several bands more than group V (infected orally, treated with antibiotic) which had one band (Fig. 1,2).

The same can be observed with groups VIII (infected i.m., not treated with antibiotic) which had several bands where VI (infected orally, not treated with antibiotic) had 2-3 bands fewer (Fig. 1,2).

This means that parentral route gave more bands in the immunoblot and stronger immune response than the oral route (deeply stained bands).

Humoral immunity were detected by micro plate agglutination test in the 1st week post treatment and varied among different groups either in percent of positivity or titer. ***Parry and Porter (1981) and Hassan et al. (1991)*** reported that titer of different IgM and IgA rapidly increased followed by sharp decrease in response to infection or vaccination, where the IgG increased later on and persisted for long period. The least positivity were recorded in the in groups V along the experiment (table 3) and varied from 11.1 % in the 1st week post treatment to 60 % in the 2nd week post challenge. The low titers in group V may be explained that oral infection elicited more local immunity in the intestine rather than circulating humoral antibodies ***(Pritchard, 1978).***

While high rate of positive sera was seen with groups not treated with antibiotics and either vaccinated or infected parentrally (i.m). The same result can be obtained with the immunoblot by comparing group VI (infected orally, not treated with antibiotic) had 2-3 bands in the immunoblot more than group V (infected orally, treated with antibiotic) (Fig 1, 2 and table, 4). Group V produced the lowest number of reacting bands that stained very faint (Figures 1-2 and table, 4). Also group VIII (infected i.m., not treated with antibiotic) had 1-3 bands more than group VII (infected i.m., treated with antibiotic) (Fig 1, 2).

The antibiotic either had abolished some important antigens to react with the immune system, or the antibiotic had affected negatively on the immune system and decreased its responsiveness to the invading organism antigens.

Also the titer of agglutinins followed the same patterns of positivity where it was detected as early as 1 week post-infection, then increased steadily then declined on the 4th week post treatment (2nd week post challenge) within the same group. ***Gast and Beard (1990); Barrow (1992) and Rana and Kulshreshtha (2006)*** reported that antibody titers peaked at 1-2 weeks post-inoculation and declined steadily, although most birds were still identified as sero-positive at 10 weeks post inoculation. While the differences among groups were highly significant (tables 2, 3), the differences between groups II, III and IV were not significant during 1st and 2nd week post treatment. Then this pattern changed and significant difference were seen between these group. By antibiotic treatment there were significant reduction in agglutinin titre in groups I, V and VII (1,1and1.85) than the analogous one, II, VI and VIII (1.68, 2.04 and 2.34) respectively.

The effect of age was evident on titer growth as there were significant increase in 1st and 2nd wk post treatment in all group except group I and V and this may be attributed to the effect of antibiotic although group VIII was also treated with the same antibiotic but not greatly affected.

This may be explained that the live salmonella vaccine (group I) is more susceptible to the antibiotic treatment than the pathogenic strain. Moreover infection by (i.m.) route elicited the highest titers among the other groups. ***Subhabphant et al. (1983)*** reported that protection was more pronounced when chickens were inoculated intramuscularly rather than orally.

Nitrocellulose membrane has been successfully used as a solid phase support for antigens in the detection of antibodies to proteins ***(Hawkes et al., 1982).***

Result of western blot testing of whole *S. enteritidis* lysate against pooled sera from each group tested at the end of 1st -5th week post-treatment revealed that no reaction could be detected in the 1st week post treatment in all experimental groups. Then by the end of the 2nd week post-treatment the sera respond positively in all groups but the number of positive band differ greatly among groups and by the end of either 4th or 5th week all group tend to reach a plateau where only 2 bands of the same molecular weight were detected (Figs 1-4). The bands appeared on the 2nd week post treatment then disappear by the 5th week. IgM class which appeared early and declined early (Figs. 1, 2), while the bands forming plateau with the same molecular weight may be the IgG class (Fig. 4) ***(Parry and Porter, 1981 and Hassan et al., 1991)***.

In a comparison between micro plate agglutination and western blot in detecting positive serum, irrespective, the treatment applied it is clear that the positivity in agglutination test ranged from 11.1% to 100 %.

Rajashekara, et al., (1999) reported that by the first week after infection 66–78% of chickens were found positive for SEF14 antibodies in the serum and the number of positive birds increased subsequently to 89–100%, this difference may explained as the author used only SEF14 protein (dot blot), while in the present paper the whole *S. enteritidis* lysate that contains a variety of proteins.

So it may conclude that serological assays may be used as preliminary screening of flocks prior to bacteriological culturing. The nitrocellulose membrane based assays have been shown to possess higher specificity for detection of antibodies to soluble proteins than micro titer plate based ELISA ***(Hawkes et al., 1982).***

Using the immunoblot test; chickens infected with *S. enteritidis* and not treated with antibiotics showed 1-3 bands more than those infected and treated with antibiotics.

Concerning the use of bacterin it is clear from table (4) that locally prepared bacterin elicited more bands than commercial bacterin. This is because the *S. enteritidis* strain in case of locally prepared vaccine and the immunoblotting antigen were homologous.

To know the immunological difference in the immnuoblot testing, between inoculation of live pathogenic *S. enteritidis* (i.m.) and inoculation of killed homologous vaccine (bacterin) we should compare between group VIII and group IV. Group VIII had 5 bands at 2 weeks post-vaccine while group IV had only 2 bands, but after challenge they were similar. Also the same notice is found with the micro titer agglutination 1 and 2 weeks post vaccination, where injected live pathogenic bacteria produced a geometric mean titer of $\log_{10}$ 2.34 and 3.32, respectively, where group IV injected with local bacterin from the same strain gave a micro agglutination titers of $\log_{10}$ 1.8 and 2.54 on the first and the second week post-vaccination, respectively. This means that live pathogenic bacteria injected i.m. produced higher mean titers than the bacterin prepared from the same strain and injected via the same route. This can be explained that preparation of bacterin may affect the quality of bacterial proteins through denaturation or the live bacteria still have the ability to reproduce. Also the difference in the immune profile between the 2 cases may be due to the bacterial by-products as toxins or other virulence factors.

It is noticed also that by the end of 4^{th} and 5^{th} week post vaccination, all groups tend to form a serological plateau seen in the immunoblot represented by fixed similar 2 band appeared in all the groups. This can be explained that in the first 2 weeks post-vaccination, the immune response is made up by IgM, which disappear and replaced later by IgG.

Table (2): Geometric mean titers of positive serum samples in micro titer plate agglutination test expressed as log $_{10}$ ± S.E.

Treatment Groups	Post-treatment* (vaccination or infection)		Post-challenge**		
	1st week	2nd week	1st week	2nd week	3rd week
	Log_{10}±S.E	Log_{10}±S.E	Log_{10}±S.E	Log_{10}±S.E	Log_{10} ±S.E
live vaccine s/c + antibiotic A (I)	1^{b} ± 0	1.15^{b}±0.15	1.9^{a} ±0.2	2.77^{a}±0.1	2.76^{a} ±0.09
live vaccine(II)	1.68^{cd}±0.07	2.22^{c}± 0.07	2.57^{b}±0.13	3.18^{ab}±0.07	3.06^{ab} ±0.11
commercial bacterin (III)	1.56^{c}± 0.06	2.42^{cd}±0.07	3.08^{c}±0.12	3.56^{bcd}±0.11	3.38^{bc} ± 0.07
local bacterin (IV)	1.80^{cd}±0.09	2.5^{cde}±0.11	3.02^{c}±0.07	3.80^{d}±0.09	3.50^{cd} ± 0.09
Infected orally + antibiotic A (V)	1^{b} ± 0	1.4^{b} ±0.12	2.15^{a} ±0.11	2.87^{a}±0.08	2.83^{a} ± 0.09
Infected orally (VI)	2.04^{de}±0.11	2.7^{de}±0.07	3.38^{cd}±0.07	3.68^{cd}±0.07	3.62^{d} ± 0.07
Infected i.m A (VII) + antibiotic A	1.85^{c}±0.7	2.87^{e}±0.1	3.1^{c} ±0.09	3.3^{bc} 0.13	3.1^{abc} ±0.06
Infected i.m (VIII)	2.34^{e}± 0.14	3.32^{f} 0.12	3.62^{d}± 0.07	3.80^{d}±0.09	3.68^{d} ±0.07
Control negative (IX)	0.00^{a}	0.00^{a}	2.66^{b}± 0.11	3.32^{bc}±0.07	3.62^{d} ±0.07

*Treated at 14 day of age

There is a highly significant difference between blocks carrying different letter in the same column at p≥ 0.01

Table (3): Percent of positive serum samples in micro titer plate agglutination test.

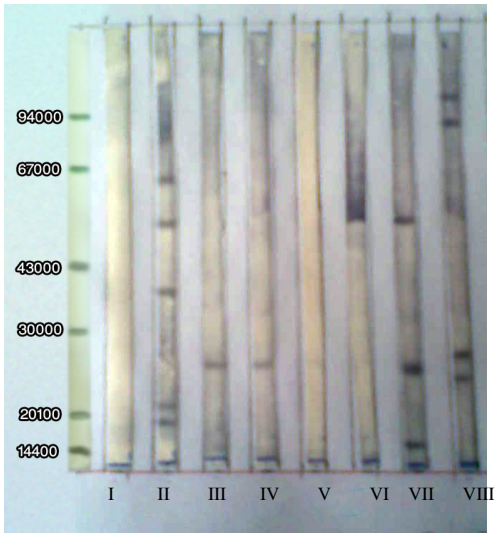
Treatment groups	Post-treated* (vaccination or infection)		Post-challenged**		
	1st week	2nd week	1st week	2nd week	3rd week
	% +ve	% +ve	% +ve	% +ve	% +ve
live vaccine s/c + antibiotic A (I)	11.1	22.2	20	60	40
live vaccine (II)	22.2	33.3	40	60	60
commercial bacterin (III)	33.3	55.6	60	80	60
local bacterin (IV)	33.3	55.6	60	80	60
Infected orally + antibiotic A (V)	11.1	33.3	20	60	40
Infected orally (VI)	33.3	44.4	40	80	60
Infected i.m A (VII) + antibiotic A	33.3	55.6	60	80	60
Infected i.m (VIII)	55.6	77.8	100	100	80
Control negative (IX)	0	0	20	60	60

*Treated at 14 day of age

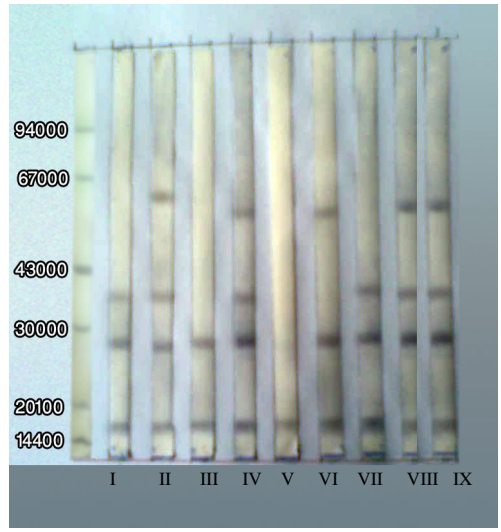
There is a highly significant difference between blocks carrying different letter in the same column at p≥ 0.01

Table (4): Total number of bands appeared in the immunoblot testing after different treatments.

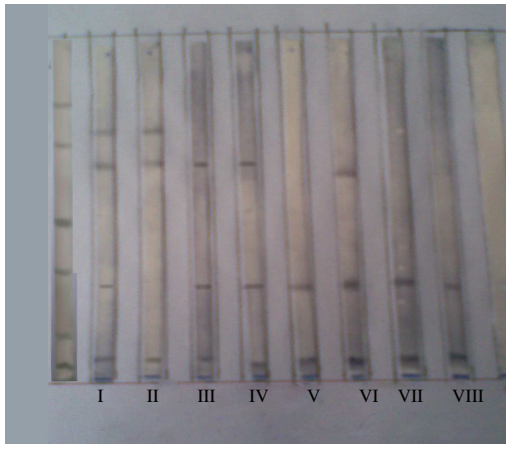
Time of testing	I	II	III	IV	V	VI	VII	VIII	IX
1ST wk post vaccine	-	-	-	-	-	-	-	-	-
2nd wk post vaccine	1	6	2	2	1	2	4	5	-
1 wk post challenge	4	5	2	5	2	4	4	5	5
2 wks post challenge	4	3	3	3	2	3	2	2	Not done
3 wks post challenge	2	2	2	2	2	2	2	2	2



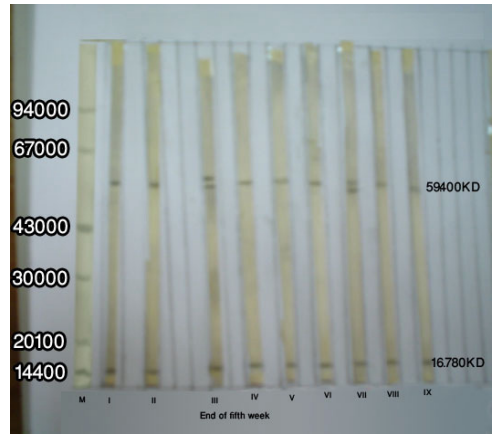
Two weeks post treatment



Three weeks post treatment



Four weeks post treatment



Five weeks post treatment

CONCLUSION

Both serum micro titer plate agglutination and immunoblot are important to understand the immune response in different immune situation for *S. enteritidis*. Immunoblot clarified the detailed action of antibiotics on the fractionated immune response to different antigens of *S. enteritidis*.

Since *S. enteritidis* live vaccines create good level of cell mediated immunity they are important to protect layers and breeders against salmonella infection. Killed vaccines generate high level of humoral immunity that is important to protect the breeder progeny chicks. Although antibiotics are important to control *S. enteritidis* shedding, contamination of the environment and organ colonization but antibiotics have side effects as they interfere with the production of agglutinins which are important for long term immunity and protection. *S. enteritidis* given parentrally produced more immune reaction bands in the immunoblot test than those given orally.

Locally prepared vaccine also produced more bands in the immunoblot than the commercial vaccines because of the homogeneity between the antigen of the test and the vaccinal strain.

Live pathogenic bacteria injected i.m. produced higher geometric mean titers and more bands in the immunoblot than the bacterin prepared from the same strain and injected via the same route.

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تقييم الاستجابة المناعية بعد العدوى الصناعية أو التحصين ضد ميكروب السالمونيللا أنتيريتيدس باستخدام اختباري الطبع المناعي أو اختبار التلازن الطبقي المصغر

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أجريت هذه الدراسة للمقارنة بين الاستجابات المناعية بواسطة اختبار التلازن الدموي أو اختبار الطبع المناعي. وأظهرت الدراسة أن اختبار التلازن استطاع تحديد 100% إيجابية في العينات المفحوصة في الأسبوع الأول بعد اختبار التحدي وفي المقابل استطاع اختبار الطبع المناعي تحديد أول نتيجة إيجابية من الأسبوع الثاني بعد المعاملات. بالمقارنة بين الأجسام المناعية وجد أن المجموعة المعالجة بواسطة التحصين الميت المحلي والمجموعة المحقونة عضلياً بالسالمونيلا أظهرت أعلى عيارية للأجسام المناعية (3,8).

أظهرت المعاملات اختلافات واضحة في الاستجابة المناعية بواسطة الطبع المناعي حيث أن الأسبوع الثالث بعد التحصين أو الأول بعد العدوى الصناعية أعطي أكثر استجابة مناعية وأكثر عدد من الحزمات المناعية. وقد كان لاستعمال المضادات الحيوية تأثير كبير في اختفاء عدد كبير من الحزم المناعية. كما كان هناك تأثير لطريقة العدوى على الاستجابة المناعية. فالعدوى عن طريق الفم أنتجت عددا أقل من الحزم المناعية مقارنة بتلك عن طريق الحقن في العضل.