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THE IMPETUS INFLUENCE OF SPIRULINA AS IMMUNOSTIMULANT ON THE RESPONSE OF PPR VACCINE IN SMALL RUMINANT

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ABSTRACT
The present study was conducted to evaluate the possible immuno-stimulatory effect of Spirulina when given at a daily oral dose of 800 mg/kg b.wt for 4 days before PPR vaccination to sheep and goats. The evaluation of immuno response was achieved through monitoring total serum proteins, albumin, globulin and antibody titre estimated by serum neutralizing test (SNT) and ELISA. Our results showed that the levels of total protein and globulin in sera of treated sheep and goats by Spirulina were increased and reached the peak at the fourth weak post vaccination. In addition, the titer of PPR antibodies in those sheep and goats showed significant increase in treated animals than in untreated ones and reached the peak at the fourth weak post vaccination. It was concluded that, Spirulina has an immuno-stimulatory effect in sheep and goats during PPR vaccination.
KEYWORDS: *spirulina, immunostimulant, PPR vaccine, small ruminant*

INTRODUCTION

Peste des petits ruminants (PPR) is a severe feverish viral disease of small ruminant characterized by mucopurulent nasal and ocular discharges, necrotizing and erosive stomatitis, enteritis and pneumonia (Ismail et al; 1995) .The Peste des petits ruminants disease is endemic in Africa and causes large economic losses every year due to the high rates of mortality and morbidity in infected sheep and goats (Joshi et al; 1996). The PPR virus (PPRV) belongs to the genus Morbillivirus in the family Paramyxoviridae (Mitra-Kaushik et al; 2001). The first attenuated vaccine developed against PPR involved using the lineage I African isolate, Nigeria 75/1(Diallo et al; 1989). The animals vaccinated with this attenuated PPRV were unable to transmit the challenge virus to animals they were in contact with. Anti-PPRV antibodies generated by this vaccine last for at least 3 years, which is the effective economic life of sheep and goats (Couacy-Hymann et al 1995).

Several types of Immunostimulants have been reported of such as, Chitin (Esteban et al; 2001), CPG oligodeoxy nucllеotides (Tassakka and Sakai; 2002& 2003), Nisin (Villamil et al; 2003), and Spirulina (Spirulina Platensis) (Ragap et al; 2012) .it's well known that Immunostimulants can enhance the nonspecific defense mechanisms and the specific immune response when they are followed by infection or vaccination (Anderson; 1992). Spirulina Platensis, is a cyanobacteria (blue-green filamentous microalga), it is characterized by high concentration of protein and amino acid contents and have been used as a source of food for long time (Annapurna et al; 1991 and Nandeesha et al; 2001) it's well known that the protein content of Spirulina Platensis is considered as complete and bioactive protein due to its content of all essential amino acids (Babadzhanov et al; 2004). Spirulina; filamentous alga is known by its content of protein (60-70%), vitamins, minerals and essential fatty acids such as palmitic acid,

linolenic acid (Lu et al; 2002; Lu and Takeuchi; 2004). Although, Spirulina Platensis is a well – known as natural antioxidant as well as immune-stimulant to humans and animals. It has fewer side effects and coincided to be more effective than synthetic products (Abdel-Daim et al., 2013 and Khan et al., 2005). The aqueous extract of Spirulina Platensis has a major impact on the immune system through increasing the phagocytic activity of macrophages and stimulating the NK cells.

Spirulina Platensis was showed to have a stimulatory effect on the production of cytokines & antibodies thus activating and mobilizing both T and B cells (Schwartz and Shklar 1987). The bioactive protein content in Spirulina Platensis has the ability to stimulate the intestinal immune system (Khan et al 2005) , enhance the resistance of aquatic animals against pathogen and the phagocytic activity of fish were enhanced by dietary supplementation of Spirulina Platensis (Duncan and Klesius, 1996; Watanuki et al 2006; Lee et al 2003; Abdel-Tawwab and Ahmad 2009). Moreover, the mononuclear phagocytic system in chicken was enhanced by supplementation with Spirulina Platensis that increasing the disease resistance potential may (Al-Batshan et al; 2001). Generally, it was found that Spirulina Platensis has an immunostimulating effect by enhancing the resistance of humans, mammals, chickens and fish to infections that may be through influencing hemopoiesis and stimulating antibodies and cytokines production. In addition, Spirulina platensis could activate macrophages, T and B cells thus stimulating the immune system (Blinkova et al 2001 and Krishnaveni et al 2013). It was found that; Spirulina Platensis is capable of enhancing the immune response by several pathways in different animals species such as in chicken (Qureshi et al, 1996) and Cyprinus Carpio Fingerlings fish where Spirulina Platensis could enhance the phagocytic and lysozyme activities confer protection against hydrophila through boosting phagocytic and lysozyme activities(Delhi Bai et al 2014). Spirulina, it induced good stimulation to the immune system and increased the resistance of humans as well as animals (including mammals, poultry and fish) through stimulating the production of antibodies and cytokines (Promya and Chitmanat 2011). Innate immune system is perfectly stimulated by dietary Spirulina Platensis (Hironobu et al; 2006).

THE AIM OF STUDY

The present work was designed to improve the potency of the locally produced live attenuated PPR vaccine in order to provide sufficient immunity for small ruminants against PPR infection.

MATERIALS AND METHODS

I – Materials:-

1 - Spirulina Platensis:-

Spirulina Platensis was obtained as a fresh powder from Arabic Academy for Science, Technology and Maritime Transportation, Alexandria. Spirulina Platensis was used for sheep and goats at a dose 800 mg/kg body weight per day for 4days per os according to Soltani et al (2012) before the vaccination with Peste des petites ruminant's vaccine.

2 - Peste des petites ruminant's vaccine:-

A Vero cells adapted PPR vaccine prepared from Nigerian 75/1 strain supplied by Veterinary Serum and Vaccine Research Institute, Abassia, Cairo was used for vaccination of experimental sheep and goats. The vaccine had a titer of 5 log 10 TC ID50 / ml. Titration of the used vaccine was done by microtitre plate according to Ferreira (1976) using Vero cell.

3 - Viral antigen:-

The supernatant fluid of infected Vero cell culture with PPR virus was collected at full cytopathic effect (CPE).The supernatant was concentrated using polyethylene glycol 6000 and used as antigen in ELISA.

II - Animals:-

Fifteen male sheep and fifteen male goats (6 months old) of local breeds were screened using serum neutralizing test (SNT) and found to be free from Peste des petites ruminants antibodies. Animals were kept in clean stable; food and water were supplied ad libitum.

III- Methods:-

1 - Vaccination of experimental animals:-

Sheep and goats were divided randomly into three groups (5 animals / group).the 1st group of each species was kept as control negative (G1) and (G4), the 2nd group of each species (G2) and (G5) was kept as control positive where animals received Peste des petites ruminants' vaccine (field dose 10^3) while, the 3rd group of each species (G3) and (G6) were treated by Spirulina Platensis for four days with the same previously mentioned dose then vaccinated directly by Peste des petites ruminants' vaccine. Serum samples were collected on days 0, 2, 4, 7, 14, 21, 28 and 35 post vaccination for estimation of serum proteins and PPR antibody titers by ELISA and serum neutralization test (SNT).

2 - Estimation of total serum proteins and globulin:-

Serum total proteins were estimated using the method outlined by Lowry et al (1951). Albumin Content was measured using a standard albumin estimation kit (Aljumhuria Company) and the globulin content was estimated by subtracting albumin values from total protein values.

3 – Determination PPR antibodies in sera of experimental animals:-

A - Serum neutralization test (SNT):-

Both screening and quantitative SNT were performed by the micro technique as described by Ferreira (1976), in flat bottom tissue culture micro titer plates containing Vero cells. The SNT was applied on sheep and goat sera before and on week intervals after vaccination with PPR vaccine. The end point neutralizing antibody titer was expressed as the reciprocal of the final dilution of serum inhibiting the CPE of 100-200 TCID50 of PPR virus according to Singh et al (1967).

B - The indirect enzyme linked immunosorbent assay (ELISA):-

It was performed to evaluate the humoral immune response according to the method previously described by Anderson et al (1982).

Statistical analysis:

The data was analyzed by ANOVA to determine the significance of differences among means (Dean et al 1994).

RESULTS

The obtained results clarified that oral administration of Spirulina platensis to sheep and goat prior to vaccination by peste des petites ruminants' vaccine increased the titers of antibodies. The immune response was evaluated through measuring the level of total protein and globulin in sera of tested animals. At the same time we measured the level of neutralizing antibodies through quantitative SNT and indirect ELISA.

A - Results of estimation of serum total protein and globulin:-

From the obtained data we noted that, the level of total protein and globulin in sera of the control positive sheep (G2) and control positive goat (G5) was 6.43±0.23 , 2.79±0.13 , 7.77± 0.27 and 3.43± 0.17 respectively as shown in table (1 and 2) in the first week . Those levels reached the peak at the fourth weak, where their average were 7.41±0.27, 3.43±0.26 (G2) , 8.56±0.26and 3.89±0.09 (G5) for total protein and globulin respectively as shown in tables (1 and 2). However, serum total protein and globulin levels of (G3 and G6) which was treated by Spirulina platensis were significantly increased (7.53±0.31 and 3.34±0.23 in G3 and 8.27± 0.18 and 3.59±0.19 in G6 respectively) as shown in table (1 and 2) in the first week and reached the peak at the fourth weak where their levels reached 8.54±0.26, 3.58±0.22 (G3), 9.13± 0.18 and 4.23± 0.17 (G6) respectively.

B - Results of determination of PPR antibody titer in sera of experimental animals:-

The obtained data showed that the levels of neutralizing antibodies in sera of the control positive sheep (G2) and control positive goat (G5) in the first week were ≤ 2 and ≤ 4 by serum neutralizing test respectively as shown in tables (3 and 4) and were 0.19±0.052 and 0.43±0.046 respectively by ELISA as shown in tables (5 and 6). These levels reached the peak at the fourth weak, where their averages were 64 by Serum neutralization test and 1.58±0.014 and 1.59±0.024 by ELISA respectively. However, the levels of neutralizing antibodies in sera of G3 and G6 which were treated by Spirulina platensis showed significant increase and reached up to ≤ 8 by serum neutralization test and 0.58± 0.063 and 0.53± 0.043 by ELISA respectively in the first week. These levels reached the peak at the fourth weak, where their average were 128 by serum neutralization test as shown in tables (3-4) and were 1.89±0.045 &1.96±0.045 by ELISA respectively as shown in tables (5 and 6) .

DISCUSSION

PPR remains to be a main socio-economically important disease of sheep and goats. The disease is characterized by high percentage of morbidity and mortality and is among the greatest threats to a successful livestock production in many parts of the world where it exists (Sarkar et al; 2003). Vaccination considers as the simplest and most logical preventive measure against infectious diseases and this practice is considered by far the most humane and cost effective method of combating the spread of diseases (Sen et al ; 2010). Outbreaks of disease have been reported among vaccinated and non-vaccinated animals due to vaccine failure as a result of rapid vaccine deterioration and poor immune response of sheep and goats to vaccination. This requires increasing the immune response of sheep and goats to vaccination by employing the simultaneous administration of an immunostimulant and PPR vaccine (Wauwe and Jassen; 1991 and Undiandeye et al; 2014). Spirulina Platensis has been used as an immunostimulant in humans and animals because it has beneficial effects on host defense mechanism and restores depressed immune responses in both animals and humans (Ravi et al ; 2010). Serum total protein and globulin are considered as good indicators for determining immune system activation (Siwicki et al., 1994). Our results showed significant increase in the concentrations of serum protein, albumin and globulin after treatment of vaccinated sheep and goats with Spirulina Platensis for four days when compared with control positive (vaccinated animals with PPR) which could be related to enhanced immune response by Spirulina Platensis. These results match with those of Bai et al, (2014).

Chu et al (2013) mentioned that supplementation of Spirulina in mouse significantly enhanced IgG level after the first vaccination but not secondary immune response following vaccination due to enhancement of the primary immune response. In addition, Feeding of Spirulina in mice has been shown to

significantly increase the IgM antibody-producing cells in the spleen during primary immune response, but had little effect on the production of IgG antibodies during secondary immuneresponse (Hayashi et al., 1994). Administration of Spirulina enhanced IgA level in humans (Ishii et al., 1999). In the present study oral administration of Spirulina Platensis resulted in significant increase in antibody titer for PPR in the sera of vaccinated sheep and goats compared to control positive sets as observed by SNT and ELIZA results. These results suggest that administration of Spirulina prior to vaccination against PPR had an Immuno-stimulatory effect increasing the antibody titer thus may playing a role in conferring significant protection and increasing resistance to PPR infection. Similar results obtained by Hironobu et al., (2006) & Ravi et al., (2010) and Ragap et al., (2012). Spirulina enhances the immune response, by increasing the phagocytic activity of macrophages as well as stimulating the NK cells. It also played a role in the activation and mobilization of T and B cells due to its stimulatory effects in the production of cytokines and antibodies that agree with Schwartz and Shklar (1987). So, it could be concluded that administration of spirulina to sheep and goat before vaccination with PPR vaccine could provide high immunization with long duration.

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Table 1-Monitoring of total serum proteins, albumen and globulin in sheep

Serum proteins (gm/dl)in sheep groups														
animals groups / parameter	(G1)	(G2)					(G3)							
							At zero Days	Spirulina treatment		Vaccination after Spirulina treatment				
		7 D P V	14 D P V	21 D P V	28 D P V	35 D P V		2 D P T	4 D P T	7 D P V	14 D P V	21 D P V	28 D P V	35 D P V
Total protein (mg/dl)	6.12± 0.30	6.43± 0.23	6.61± 0.34	7.02± 0.33	7.41± 0.27	7.32± 0.21	6.43± 0.28	6.79± 0. 25	7.29± 0.34	7.53± 0.31	7.89± 0.24	8.23± 0.22	8.54± 0.26	8.41± 0.21
Albumen (mg/dl)	3.29± 0.36	3.72± 0.11	3.90± 0.14	3.98± 0.13	4.06± 0.18	3.98± 0.23	3.89± 0.18	3.98± o.27	4.10± 0.20	4.10± o.18	4.28± 0.21	4.41± 0.17	4.50± 0.20	4.47 ± 0.25
Globulin (mg/dl)	2.63± 0. 16	2.79± 0.13	3.02± 0.20	3.21± 0.19	3.43± 0.26	3.40 ± 0.21	2.54± 0. 32	2.81± 0.13	3.17± 0.24	3.34± 0.23	3.65± 0.16	3.53± 0.26	3.58± 0.22	3.51± 0.18

Treatment means did not differ significantly (P < 0.05)
G1 = control negative
G2 = Control positive (vaccinated with PPR)
G3 = Treated group with Spirulina then vaccinated with PPR
D P V = Days Post vaccination
D P T = Days post treatment

Table 2-Monitoring of total serum proteins, albumen and globulin in goats

Serum proteins (gm/dl)in goats groups														
animals groups / *parameter*	*(G4)*	*(G5)*					*(G6)*							
							At zero Days	Spirulina treatment		Vaccination after Spirulina treatment				
		7 D P V	14 D P V	21 D P V	28 D P V	35 D P V		2 D P T	4 D P T	7 D P V	14 D P V	21 D P V	28 D P V	35 D P V
Total protein (mg/dl)	7.35± 0.34	7.77± 0.27	7.98± 0.35	8.40± 0.28	8.56± 0.26	8.54 ± 0.21	7.22± 0.28	7.54± 0.32	7.79± 0.36	8.27± 0.18	8.64± 0.21	8.89± 0.31	9.13± 0.18	9.07± 0.24
Albumen (mg/dl)	3.92± 0.33	4.15± 0.31	4.21± 0.29	4. 46± 0.28	4. 61± 0.25	4.59± 0.27	3.87± 0.27	4.16± 0.18	4.31± 0.19	4.47± 0.22	4.50± 0.25	4.72± 0.19	4.68± 0.21	4.71± 0.19
Globulin (mg/dl)	3.30± 0. 19	3.43± 0.17	3.61± 0.21	3.82± 0.16	3.89± 0.09	3.80± 0.16	3.18± 0.19	3.27± 0.24	3.43± 0.27	3.59± 0.19	3.91± 0.22	4.15± 0.17	4.23± 0.17	4.19± 0.21

Treatment means did not differ significantly (P < 0.05)
G4 = control negative
G5 = Control positive (vaccinated with PPR)
G6 = Treated group with Spirulina then vaccinated with PPR
D P V = Days Post vaccination
D P T = Days post treatment

Table -3 Serum neutralizing antibody titer in sheep

	Mean PPR serum antibody titer /day post vaccination				
Animals groups	7 D P V	14 D P V	21 D P V	28 D P V.	35 D P V
G1	0	0	0	0	0
G2	≤ 2*	≤4*	16*	64*	64*
G3	≤8*	≤ 16*	64*	128*	128*

***Antibodies titer =the reciprocal of serum dilution which neutralize and inhibit the CPE of 100-200 TCID50 OF PPR VIRUS.**

G1 = control negative
G2 = Control positive (vaccinated with PPR)
G3 = Treated group with Spirulina then vaccinated with PPR
D P V = Days Post vaccination

Table -4 Serum neutralizing antibody titer in goats

	Mean PPR serum antibody titer /day post vaccination				
Animals groups	7 D P V	14 D P V	21 D P V	28 D P V	35 D P V
G4	0+	0	0	0	0
G5	≤4*	8*	32*	64*	64*
G6	≤8*	≤ 32*	≤ 64*	128*	128*

***Antibodies titer =the reciprocal of serum dilution which neutralize and inhibit the CPE of 100-200 TCID50 OF PPR VIRUS.**
G4 = control negative
G5 = Control positive (vaccinated with PPR)
G6 = Treated group with Spirulina then vaccinated with PPR
D P V = Days Post vaccination

Table 5 PPR ELISA antibody titer in sheep

	Mean PPR serum antibody titer /day post vaccination				
Animals groups	7 D P V	14 D P V.	21 D P V	28 D P V	35 D P V
G1	0	0	0	0	0
G2	0.19±0.052	0.42±0.045	1.27±0.014	1.58±0.014	1.56±0.091
G3	0.58± 0.063	1.39±0.068	1.66±0.051	1.89±0.045	1.88±0.050

Treatment means did not differ significantly (P < 0.005)
G1 = control negative
G2 = Control positive (vaccinated with PPR)
G3 = Treated group with Spirulina then vaccinated with PPR
D P V = Days Post vaccination

Table 6 PPR ELISA antibody titer in goats

	Mean PPR serum antibody titer /day post vaccination				
Animals groups	**7 D P V**	**14 D P V**	**21 D P V**	**28 D P V**	**35 D P V**
G4	0	0	0	0	0
G5	0.43±0.046	0.57±0.049	1.37±0.034	1.59±0.024	1.57±0.051
G6	0.53± 0.043	1.41±0.048	1.56±0.051	1.96±0.045	1.93±0.054

Treatment means did not differ significantly (P < 0.005)
G4 = control negative
G5 = Control positive (vaccinated with PPR)
G6 = Treated group with Spirulina then vaccinated with PPR
D P V = Days Post vaccination