Original ARTICLE Mirror of Research in Veterinary Sciences and Animals MRVSA/ Open Access DOAJ

Humoral immune response assessment against different hemorrhagic septicemia vaccines in local buffaloes in the marshes of southern Iraq

Khalid Al-fartosi [1], Jalil Abed Gatie [2 *], Saad Abdul Azeez Atiayh [3]; Amir Musa Yasein [4]

[1] College of Sciences / University of Thi-Qar, [2] Veterinary directorate / Ministry of Agriculture, [3] College of Medicine / University of Thi-Qar, [4] Veterinary directorate, Ministry of Agriculture

ARTICLE INFO

Received: 14.08.2020
Revised: 20.09. 2020
Accepted: 24.09.2020
Publish online: 30.09.2020

***Corresponding author:**
Jalil Abed Gatie: Email address: jalelabed@yahoo.com

Abstract

Hemorrhagic septicemia (HS) is an important disease in cattle and buffaloes and lead to great economic losses in Asia and Africa. HS gains a great importance and attention in Iraq, because it is one of the most dangerous diseases in buffaloes than cows. Vaccination program was established since 2008 for buffaloes breeders in the marshland in southern of Iraq. Consequently, this study was designed to reassess the vaccination program and evaluate the humor immune response in buffaloes vaccinated by two types of HS vaccine in the marshlands south of Iraq. The study was conducted a challenge examination on buffaloes directly with a study of some physiological and immunological aspects before and after examination of the challenge test using an indirect haemagglutination test. The study also evaluated the effect of the virulent bacterium on the animal's body depending on the clinical symptoms and postmortem pathological changes. The results of this study approved that a single dose of oily vaccine can guarantees the protection of animals more than six months , moreover it appeared better than two doses of alum sediment vaccines in terms of immune strength and immunological duration. In conclusion this study approved the ability of single dose of oily vaccine to protect buffaloes for long period. Therefore, the authors recommend to use this vaccine for protection of buffaloes from HS instead of alum precipitant vaccine.

To Cite this article: Khalid Al-fartosi , Jalil Abed Gatie , Saad Abdul Azeez Atiayh ; Amir Musa Yasein. (2020). Humoral immune response assessment against different hemorrhagic septicemia vaccines in local buffaloes in the marshes of southern Iraq. (2020). MRVSA. 9 (2): 25-42. Doi: http://dx.doi.org/10.22428/mrvsa-2020-0092-03

Keywords: Buffaloes, H.S., *Pasturella multocida* , Vaccine, Marshes , Iraq

Introduction

Hemorrhagic septicemia is a wide spread disease that affect cows and buffaloes, and cause catastrophic epidemics in countries of Asia and Africa leading to high rates of injuries and deaths. The disease has been recorded in wild colonies in many Asian and European countries. Epidemics often occur during the season of high humidity. In an analysis of diseases in India for the period 1979-1986, HS was found to cause the highest mortality and the second highest incidence in cattle and buffalo compared to foot and mouth disease, anthrax and occasional anthrax, respectively (Benkirane, and DE ALWIS, 2002; Chandrasekaran *et al.,*1994). The pathogen, *Pasterulla multocida* is Gram-negative, which is coexist in the nose and pharynx area of buffalo. The disease can occur after exposing the animals to some predisposing environmental factors that help in spread of the disease such as high temperature, crowding, bad ventilation, transport, and malnutrition. There are different serotypes, the asian serotype (B: 2) and the African serotype (E: 2) (Carter and Huddleston classification). According to the (Namioka and Carter classification), two similar strains the 6: B and 6: E are considered as the two main causes of disease. The serotype (B: 2,5) is predominant, while the serotype (B: 3,4) is also recorded in the falou deer (Albaek *et al.,* 2009). Similar cases of hemorrhagic sepsis caused by (A: 1, A: 3) in cows and buffaloes have been recorded in India. The geographical distribution of the disease includes also some regions of Asia, Africa, the Middle East, and southern Europe (Muneer *et al.,* 1994).

Postmortem examination of most dead animals from the disease shows severe swelling in the neck resulting from the accumulation of high amounts of fluids with blood. There are profuse hemorrhagic spots in many tissues and organs, especially in the lining membranes. Chest, endocardia and abdominal cavities may contain blood serous fluids. The lung is clearly congested with fluid accumulation, and in general there is a foam in the nasal cavity, trachea and bronchioles, as microscopic examination shows the presence of interstitial lung inflammation and pulmonary shear, in addition to the clusters of neutral white cells and phagocytic cells in many tissues. All postmortem changes are similar to what is observed in severe mold and rotting shock. Severe epidemiological cases occur in the endemic and non-endemic areas of the disease. The disease may occur as secondary complications in cattle and buffalo after the spread of foot and mouth disease. The mortality rate of the disease may reach 100% if treatment is not done in the early stages (Jaffri *et al.,*2006; Mustafa *et al.,*1978).

Humoral immunity plays an important role in protecting against disease. Moreover, preventive immunizations are the best way to control HS (Qureshi and Saxena, 2014). The basic vaccines required to be used against hemorrhagic sepsis include three types: the live vaccine from non-harmful strain of bacteria, alum vaccine and oily vaccine. Vaccination with live bacterial needs to be re-pollinated then the injection in a large amount may cause shock and disease symptoms. Conversely, these events are less with vaccines deposited with alum and are not present in the oily vaccine (Rosen, 1981). The bacterial vaccine has been used in (Mayanamar) since 1989 as a nasal spray, but it has not been used in other countries and killed vaccines are the only vaccines used in countries affected by the disease (Priadi and Natalia, 2001).

Review of literature revealed few published articles regarding many pasteurellosis outbreaks in buffaloes and cattle in southern Iraqi marshes. (Al-Hamed, 2010; Salah, 2012; Al-Shemmari, 2013; Waffa *et al.,* 2014). However, scarce information is available regrading assessment of vaccination program used in this area for protection of buffaloes. Therefore, this study was designed to evaluate the vaccination program in buffaloes and to measure humoral immune response in buffaloes vaccinated by two types of HS vaccine challenged with virulent bacterium in the marshlands south of Iraq

Materials and methods

Experimental animals

Twenty four buffaloes from both sex (Males and females divided randomly), age of 3-6 months were used in this study. The buffaloes entered into a pre-closed, tightly prepared enclosure equipped with appropriate livelihoods for buffaloes and adjacent to a small river branching from the Euphrates River. All convincing environment conditions were available for rearing buffalo, as the river contains a good cover of reeds and other plants, in addition to Buffalo diving swamp acts as a coolant. The animals were numbered and all information were recorded. The animals health and behavior were observed for a month and treated against external and internal parasites. These animals were validated to conduct research, and they vaccinated against foot-and-mouth disease.

Eight health local rabbits previously unvaccinated against the bacterium were used to inoculates and maintain the virulence bacterial strain, that would be used during the challenge test.

Blood samples were collected from all experimental animals after acclimatization (one month after they came to the barn) in vacutainer without anticoagulant tubes. The serum was separated and frozen under - 20 ° C until use for further investigations. The antibacterial antibodies were examined using an indirect hemagglutination test.

The precipitating antigen (particulate antigen) and soluble antigen were prepared in the laboratories of Al-Kindi company for production of animal vaccine in Iraq, according to the method described previously (Manual OF Diagnostic Tests and Vaccines, 2008).

The stuck red blood cells were prepared according to the method of Carter (Carter, 1955) that used for indirect hemagglutination test.

The used vaccines were prepared at Al-Kindi Company for the production of vaccines and veterinary medicines. While the challenge dose was prepared from the virulent strain of the *Pasterulla multocida* bacterium used in the production of the hemorrhagic septicemia vaccine in Al-Kindi company. Bacterial count was done and injected at a concentration of 10^9 bacteria / cm^3 subcutaneously for each animal in the first challenge test and with a concentration of 10^8 microbes / cm^3 subcutaneously for each animal in the second challenge .

Immunological tests

Venous blood samples from experimental animals were collected using sterile ,vacuum tubes. Samples left for half an hour, then placed in a cooled container and transferred to the laboratory. The serum was isolated and frozen under -20 C° until examination. An indirect hemagglutination test was carried out on the samples a month after their presence in runway according to Shayegh *et al.,* (2010). In all samples of the experimental animals, immune antibodies of the *Pasterulla multocida* were found at day 150 before experiment. Accordingly, the experiment was postponed and the blood samples were withdrawn after 90 days, where the presence of immune antibodies also continued. The animals were left to complete 180 days, which is the known limit for the survival of the immune antibodies after vaccination of the animals, then blood samples were collected and examined by the indirect hemagglutination test (considered as zero day results). Laboratory samples were continued to monitor the immune response to the end of the experiment.

Animal vaccination and group distribution

The animals were divided into four groups (each group consists of 6 animals) as follows:

Group (I): vaccinated by Precipitated alum vaccine in a dose of 3 cm^3 (HS / APV) subcutaneously in two doses between them for a period of (21) days. The numbers included 1, 3, 30, 4, 27, 62

Group II (II): Vaccinated with a mixture of vaccines, with a dose of 3 cm^3 and occasional dose of 2 cm^3, precipitated alum (HS + Bl / APV) subcutaneously in two doses between them for a period of (21) days. The numbers included 10, 12, 24, 11, 13 , 21.

Group III: vaccinated intramuscular with oily HS vaccine (OAV) at a dose of 3 cm^3 once and only, and included the numbers 6, 9, 23, 5, 7, 22.

• Group IV (Control group): It was injected with normal saline 3 cm^3 sub cut. The numbers included 14, 16, 28, 8, 15, and 25 as illustrated in Table (1).

Table (1): The division of the studied animals into groups

Groups	Types of vaccine	First challenge	Second challenge
I	APV /HS	1, 3, 30	4, 27, 62
II	APV /HS +BI	10, 12, 24	11, 13, 21
III	Oily HS	5, 7, 22	6, 9, 23
IV	Normal saline	14, 16, 28	8, 15, 25

Immunological strength tests

Indirect hemagglutination test was used in this experiment. The test was done according to Carter *et al.,* (1955) and carried out on all samples before and after the vaccination procedures, and before and after the challenge examinations.

Challenge test

The groups were re-divided, each group into two parts, and each section consisted of three animals, in order to use each section for each challenge examination. The challenge examination was carried out in two phases. The first was 21 days after the second vaccine dose (day 42). The second challenge examination was more than six months after the first vaccine dose. The results were recorded in the two tests and the effect of the challenge dose on animals was visualized. The control group divided into two parts. Three animals were considered positive control. They received a dose of the virulent bacterium during the examination of the first challenge. The other three were considered negative control who received normal saline during the same examination and these animals were used as a control group during the second challenge examination.

Time of the first challenge test

The general condition of the animals was observed, and the body temperature of all animals was recorded before the challenge examination to assess the health status and blood samples were collected. The bacterium was given to three animals at a dose of 3 cm^3 (in the manner of subcutaneous administration in the neck) from the groups vaccinated with the three types of vaccines (the first set of numbers 1- 30 -3) (the second group of numbers 10- 12- 24) (the third group of numbers 5- 7 -22). The bacterium was given at the same dose to three animals from the control group, numbers 14 -16 -28. The rest of the control group was given 3 cm^3 of physiological saline, which are numbers 8-15 -25.

Implementing the second challenge test

The bacterium was given to three animals from the groups vaccinated with the three types of vaccines (the first group of numbers 4- 27- 62) (the second group of numbers 11-13-21) (the third group of numbers 6-9-23).

The bacterium was given at the same dose to three animals from the control group, which are the numbers (8-15-25). For the purpose of ascertaining the virulence of the bacterium and confirming its action, the bacterial culture itself was given at a dose of 0.1 cm^3 to three rabbits, in good health and previously unvaccinated against the bacterium, in the femoral fold and placed under supervision.

Environment protection

A hole (before the challenge examinations were performed at the time) was prepared at a depth of 1.5 m in which the dead animals were buried so that they could not be excavated or stripped and medical materials were prepared to sterilize the dissection area and all the regular and medical tools and supplies used.

Statistics

The results were analyzed statistically using the statistical analysis program , SPSS Version 17, L.S.D., ($P \leq 0.05$). Capital letters in English were used horizontally to denote

the differences between groups. Small letters were used vertically to denote the differences between days during the experiment..

Results

The results of indirect hemagglutination test revealed a high concentration of hemagglutination antibodies titration in standard test of all serum samples collected from experimental animals at days 150 before experiment with significant increase in the third group compared to the other groups. Moreover, the titer of antibodies were showed insignificant increased on day 60 th before the experiment in the first group in compare to the second and third groups. However, this increasing was significant compared to the fourth group. The results also revealed a decrease in the antibodies titer at day zero compared to the previous two periods and without a significant difference between the experimental groups according to the type of vaccine (Table.2). The referee awarded the standard volumetric number of units of agglutination per unit volume.

Table.2: Shows the results of an indirect hemagglutination test prior to performing the experiment

Groups / **Days**	**G I**	**G II**	**G III**	**G IV**	**L.S.D**
Day 150 before experiment	28.66±20.01 ab	10.66±4.77 b	65.66±41.02 a	8.0±2.68 b	28
	B	B	A	B	
Day 60 before experiment	59.33±39.57 a	36.0±9.63 a	46.0±18.35 ab	24.66±4.88 a	27.4
	A	AB	AB	B	
Day 0	5.33±0.84 b	5.33±0.84 b	5.33±0.84 b	5.66±1.08 b	2.21
	A	A	A	A	
L.S.D	38.25	12.78	34.55	6.29	

First challenge test

The level of the antibodies increased significantly, on day 42 during the examination of the first challenge, i.e. three weeks after the second dose of the vaccine for the animals of the first and second groups compared to the third group that injected with a single dose of oily vaccine and the control group. The results showed fluctuation in the level of immunity during the days 72-102 -132-162-180 (Table. 3). Moreover, Figure. 1 also shows the pathway of the immune level during the trial period for all groups and for all animals. The results of indirect hemagglutination test from day 150 before the first

experiment to day 180 of the experiment showed the presence of immune antibodies before the experiment and drop the immune level in the zero-day. The level of antibodies increased at day 21 and 42 with fluctuation until the day 180.

Table .3: Shows the results of the indirect hemagglutination test - during the first challenge test.

Groups **Days**	**G I**	**G II**	**G III**	**G IV**	**L.S.D**
Day 150 before experiment	8.0±4.0 b	18.66±7.05 ab	24.66±19.74 ab	8.66±4.05 b	10.62
	BC	AB	A	B	
Day 60 before experiment	106.66±74.66 a	34.66±16.22 a	49.33±39.48 a	17.33±8.11 a	56.8
	A	B	B	B	
Day 0	5.33±1.33 b	6.66±1.33 b	5.33±1.33 b	5.33±1.33 b	1.75
	A	A	A	A	
Day 21	4.66±1.76 b	24.0±20.0 ab	9.33±3.52 b	10.66±2.66 ab	21.79
	A	A	A	A	
Day 42	14.66±8.74 b	11.33±4.66 ab	9.33±3.52 b	9.33±3.52 b	6.61
	A	A	A	A	
Day 72	4.0±2.30 b	8.0±0.0 ab	10.66±2.66 b	0.0±0.0	4.63
	B	Ab	A		
Day 102	32.0±18.47 b	32.0±18.47 ab	10.66±10.66 b	0.0±0.0	28
	A	A	A		
Day 132	8.0±4.61 b	16.0±9.23 ab	6.66±4.80 b	0.0±0.0	7.49
	B	A	B		
Day 162	5.33±2.66 b	10.66±5.33 ab	6.66±4.80 b	0.0±0.0	5.03
	B	A	AB		
Day 180	13.33±9.61 b	12.0±10.06 ab	8.0±4.61 b	0.0±0.0	9.64
	A	A	A		

L.S.D	60.43	27.73	35.88	7.84	

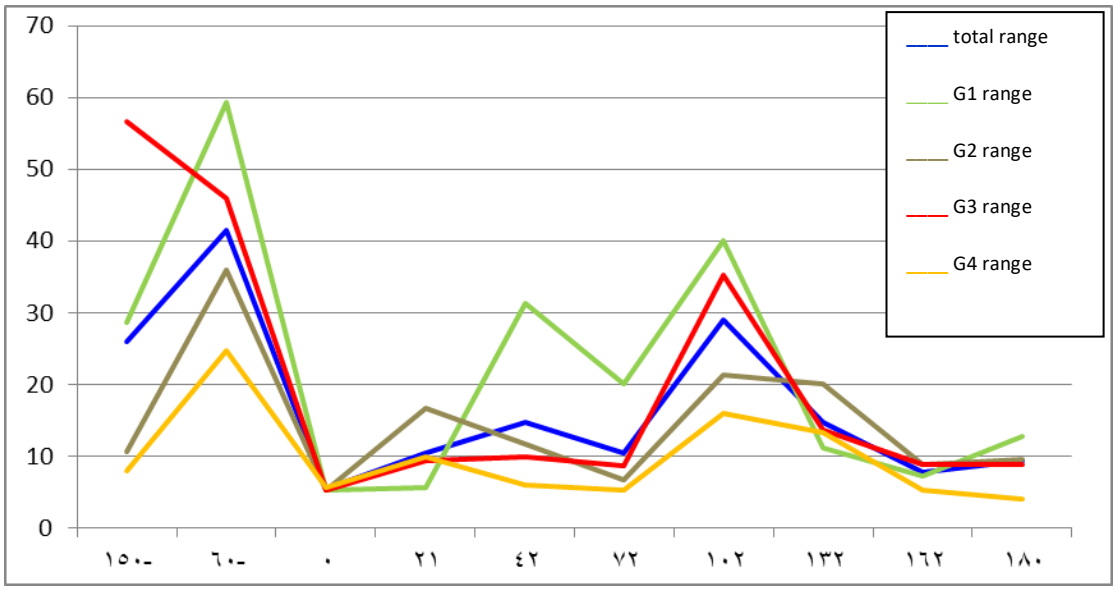

Figure. 1: The results of indirect hemagglutination test for all animals before and during the experiment.

The results of the first challenge showed the death of all animals of the positive control group (the first group) in the second day that revealing 100% mortality rate. While, one of the animal from mixed vaccine group (the second group) was died after six days of the challenge with 33.3% mortality rate. Moreover, eight out of nine vaccinated animals were survived the challenged with virulent bacteria revealing 88.8% survival rate though their suffering from severe inflammation at the injection site. The animals also suffered from high body temperature and elevation in other vital signs. These clinical signs were also recorded on the animals in the positive control group. The results of experimental study revealed the following clinical signs on the affected animals; animals appeared shock, sub normal temperature , drooping head ,fluid coming out of the mouth and nose, lethargy, complete loss of appetite, lying down, severe congestion of the eyes mucous membranes, swollen of pre-scapular lymph nodes, rapid heartbeat and breathing and depression. Moreover, before death all animals suffered from, difficult breathing and hearing bronchitis and pneumonia, severe painful swelling at the site of bacterial injection in the neck, hard and hot, swelling of the throat was not noticed clearly in the three animals. Before death the animals showed reduction in the heartbeat (44 / minute), reduction in the animal's body temperature 37 C^0 and heard rumbling sounds before the death of animals, a distinctive sound of hemorrhagic septicemia in buffaloes. The following clinical signs were recorded in the three vaccinated groups (Table .4).

Group I (HS / APV)

The group showed drowsiness, fatigue, and a partial interruption of eating on the first and second days, and on the third day it returned to its activity with the appearance of a swelling at the injection area with dimensions of 10 x 10 cm except for animal number

30 as it appeared lameness and the swelling increased and extended to the shoulder and to the neck area and then died on the sixth day. He was submitted to postmortem anatomy .Samples of blood and body fluids were collected to confirm and isolate the pathogen.

Group II (HS + Bl / APV)

This group showed drowsiness, fatigue, and a partial interruption of eating on the first and second days, and on the third day it returned to its activity with the appearance of a swelling at the injection area 10 x 10 cm except for animal number 12 where it increased to 12 x 15 cm, but all disappeared after several days.

Group III (OAV)

This group showed the lowest and fastest invisible local reaction.

Postmortem anatomy of Group I animals 14,16 and 28

The animals revealed swelling of the injection area of the neck, severe congestion of mucous membranes of the eyes, enlarged pre-scapular lymph nodes, sunken eyes, slight enlarged of throat region, hemorrhage in the spleen, clotted blood, severe congestion in the lungs, congestion and bleeding in the liver and inflammation of the subcutaneous tissue in the neck accompanied by inflammatory serous fluid.

Clinical symptoms and postmortem of animal No. 30

The animal revealed development of swelling at the fifth day after conducting the first challenge examination on the animal. The swelling was extended and covered the neck area and part of the armpits. On the sixth day, the animal revealed severe progressing of clinical symptoms including congestion in the eyes, hearing a rattle breathing, and extended swelling to the entire head, chest, and front legs with inability to stand and prolapsed head, then animal rest with strong snoring sounds, saliva flowing from the mouth and fluids from the nose. Eventually, the animal was died in the evening of the same day. This animal was showed typical clinical signs of the disease and considered as a model due to its long-term survival that allowed for the observation of pathological changes in postmortem examination. The animal revealed the following gross pathological changes: Inflammation and swelling of the subcutaneous tissue accompanied with accumulation of yellow blood serous fluids; Severe inflammation of the lungs, black spots on the surface and serous fluid in the chest cavity; Hemorrhagic spots on the heart muscle (Petechial hemorrhage) and blood clots in the heart only; Laryngitis and tracheitis; Severe congestion of the small intestine and mesenteric lymph nodes. Samples were collected from heart blood and thoracic cavity and subcutaneously fluid for bacterial culturing and confirmed the presence of the bacterium.

Body Temperature

All animals injected with virulent bacterial challenge dose during the experiment, however, the first post examination challenge revealed insignificant increased in

animal's body temperature. On day 4 and 5, the animals body temperatures were raised between 1.3 - 2.3 degrees Celsius higher than normal rates (the body temperatures were measured every day at morning and evening on day 1 and 2 before the challenge examination that appeared on day 3 in the evening. The measurement of body temperature continued up to ten days after the challenge test, and then falling to normal rates compared to negative control animals. As for the positive control animals (who received the challenge dose), they died before recording the high body temperature after the challenge. There were no clear differences in temperature or of statistical significance between animal groups except in animal No. (30), where it reached 42 C^o on the fourth day after the challenge, and then chilled. Table (4) shows the results of temperature values .

Table. 4: Shows the results of the temperatures before and after examining the first challenge test

Groups **Days**	**G I**	**G II**	**G III**	**G IV**	**L.S.D**
Day 1	39.23±0.03 c	38.96 ±0.26c	39.60±0.45b	38.60 ±0.45a	0.72
	AB	AB	A	B	
Day 2	38.50±0.32 c	38.43±0.23c	39.10±0.37b	39.16±0.60ab	1.06
	A	A	A	A	
Day 3 before the first challenge	38.83±0.20 c	38.90±0.11c	39.23±0.44b	38.33±0.13bc	0.67
	A	A	A	A	
Day 4	40.63±0.03ab	40.66±0.12a	40.90±0.05ab	38.06±0.06c	0.64
	A	A	A	B	
Day 5	41.10±0.49 a	40.16±0.26ab	41.20±0.32a	38.83±0.03b	0.85
	A	B	A	C	
Day 6	41.10±0.55 a	39.93±0.06b	40.63±0.57ab	38.93±0.13ab	1.06
	A	AB	A	B	
Day 7	40.40±0.80ab	39.83±0.29b	39.66±0.37b	38.66±0.17bc	1.24
	A	A	A	A	
Day 8	39.53±0.14 bc	39.73±0.31b	39.86±0.44b	39.20±0.32ab	0.74
	A	A	A	A	
Day 9	40.10±0.05 b	40.16±0.21ab	40.53±0.65ab	38.76±0.39b	0.46
	A	A	A	B	
Day 10	39.40±0.17bc	40.66±0.03a	40.30±0.52ab	39.86±0.29ab	2.14
	A	A	A	A	
Day 11	40.0±0.03bc	40.30±0.60ab	40.06±0.46b	38.76±0.23b	0.3
	A	A	A	B	

Day 12	39.33±0.08bc	39.70±0.05b	39.63±0.35b	39.13±0.06ab	0.24
	B	A	AB	B	
Day 13	40.0±0.05bc	40.16±0.33	40.10±0.58b	38.93±0.06ab	0.17
	A	A	A	B	
L.S.D	0.87	0.65	1.1	0.68	

Second challenge test

A significant difference in the level of immunity between the second group and other groups was observed in the indirect hemagglutination test during the examination of the second challenge. The lowest immune level was recorded on the 150th day before the experiment. The immune level increased significantly at day 21 and day 42 of the trial compared to day zero. The immune level fluctuated during the days 72-132-102-162. It decreased significantly, on day 180, the day of the second challenge examination (Table .5). The second challenge test was carried out six months after the vaccination with the booster dose (and seven months after the dose of the oil vaccine), but using a challenge dose with a concentration lower than the first challenge (10^8) bacteria / cm^3 to rely on clinical symptoms only without animal destruction. The results were as follows:

Control group

In control group, one of the animals was died after 10 days of injection with the challenge dose. The postmortem was done and the bacterial isolation was done to verify the cause. The second animal showed a localized reaction of a swelling 5 x 7 cm for a period of 14 days. While, the third animal showed a simple swelling in the neck and then disappeared after several days.

Table. 5: Shows the results of the indirect hemagglutination test during the examination of the second challenge test.

Groups **Days**	**G I**	**G II**	**G III**	**G IV**	**L.S.D**
Day 150 before experiment	49.33±39.48 a	2.66±0.66 c	88.66±83.68 a	7.33±4.37 c	60.9
	AB	B	A	B	
Day 60 before experiment	12.0±4.0 a	37.33±14.11 a	42.66±10.66 b	32.0±0.0 a	23.81
	B	A	A	A	
Day 0	4.0±0.0 a	4.0±0.0 c	5.33±1.33 b	6.0±2.0 c	3.15
	A	A	A	A	

Day 21	6.66±1.33 a	9.33±3.52 bc	9.33±3.52 b	2.66±2.66 c	7.64
	A	A	A	A	
Day 42	48.0±40.0 a	12.0±4.0 bc	10.66±2.66 b	2.66±0.66 c	26.49
	A	B	B	B	
Day 72	29.33±17.48 a	5.33±1.33 c	6.66±1.33 b	5.33±1.33 c	11.69
	A	B	B	B	
Day 102	34.66±16.22 a	21.33±5.33 b	42.66±10.66 b	16.0±0.0 b	26.27
	AB	AB	A	B	
Day 132	10.66±2.66 a	17.33±8.11 bc	16.0±0.0 b	13.33±2.66 b	7.2
	A	A	A	A	
Day 162	6.66±1.33 a	4.0±0.0 c	8.0±0.0 b	5.33±1.33 c	2.47
	B	C	A	BC	
Day 180	8.0±4.0 a	4.0±0.0 c	6.66±1.33 b	4.0±0.0 c	5.54
	A	A	A	A	
L.S.D	47.36	13.88	65.7	4.95	

Group of (HS/PAV)

One animal showed a local reaction (5 x 7) cm diameters for a period of 7 days, and the other (2 x 2) cm for a period of 7 days, and the third showed no interaction.

Group of mixed vaccine (HS+BL/PAV)

a local reaction (5 x 7) cm appeared on one of them and the other (2 x 2) cm for 5 days, and the third showed no interaction.

Oily vaccine group

A local reaction (2 x 2) cm appeared for 5 days on one of the animals, and the other two animals showed no interaction.

Discussion

The indirect hemagglutination test was used in this study as a serological test to measure the immune strength and the level of antibodies against the bacterium in experimental animals during the duration of the research. The challenge test was used as the principle test to assure the efficiency of the vaccine in protecting animals from virulent bacteria. This test was also used previously to study the effect of different factors on the efficacy of hemorrhagic septicemia vaccines (Sarwar *et al.,*2015). Many

tests was used previously to detect the strength of immune response in animals vaccinated against HS (the alum and oily vaccine) such as indirect hemagglutination test in compare to passive rat protection (PMPT (Jaffri *et al.,* 2006)

In this study, the serum samples collected from all animal's groups before experiment revealed high levels of antibodies in the indirect hemagglutination test. These results led to postponed the experiment for 6 months until declined the levels of antibodies. This result approved that these animals were previously exposed to the bacteria and developed immunity since this bacteria establish naturally in the upper respiratory system (Karimkhani *et al.,* 2011; Shayegh *et al.,* 2010). The results of this study are compatible with previous studies (Karimkhani *et al.,* 2011) that revealed the bacterial isolation from calves of buffaloes and cows. Thought, the weakness of immune system in young animals allowed the establishment of pathogenic bacteria in the nose, throat and tonsils that directed the development of antibodies against this bacteria (Ashraf et al.,2011). Another study was also approved the presence of high levels of antibodies in pre experimental samples because of previous exposure. infections or vaccinations (Ali *et al.,* 2000). The results of the current study were in agreement with the results of previous investigations (De Alwis *et al.,*1990). They showed that 32 out of 75 buffaloes were exposed to the bacterium (*P. multocida*) and these animals were turned into immune carriers. Moreover, the bacterium was also isolated, besides antibodies were found subsequently at 360 days. Rather, a high titer of antibodies were detected after 150 - 180 days after exposure to the bacteria. The results this study are also compatible with others investigators that reported a number of buffaloes showed a high level of immunity with ELISA and indirect hemagglutination tests before vaccination by a hemorrhagic septicemia vaccine, although these animals lived in an area free of disease for a period of ten years prior the experiment. The study stated that the relationship between the level of immunization and the results of the indirect agglutination test are not equal (Chandrasekaran *et al.,*1994). However, another study identified that the values of the indirect agglutination test were six times higher in healthy buffaloes than in an infected buffaloes (Farooq *et al.,* 2011).

The experimental circumstances of this study in regards to the region revealed the history of exposure to the epidemic seven years ago. Additionally, this disease considers as endemic and appears in foci from time to time over the years. These facts explain the presence of antibodies in serum of the large number buffaloes on day zero of the experiment by indirect hemagglutination test. This study justified the risen in the antibodies by the possibility of subclinical infections as in animals (7, 23, 62) in the first examination before vaccination (day 150 before the experiment) and animals (3, 7, 9, 12 , 13). These results are compatible with previous study that showed the animals in the carrier status, where bacteria found active and latent. Since, the bacteria found active in short and intermittent periods, while remaining latent for long periods and settle in the tonsils that considered as the storehouse of these bacteria for a long time (De Alwis *et al.,*1990; Rosen, 1981)

The results of this study revealed the level of antibodies in vaccinated animals of the first and second groups (alum precipitated) increases with the speed of their descent. In animals vaccinated with oily vaccine, the level of antibodies were slow and almost stable for a longer period. It was the highest level of the antibodies in the animals of the first

group (HS/PAV), then the second group (HS+BL/PAV) and finally the third group (oily vaccine). However, by comparison with the results of the challenge test, the lowest level of the antibodies was with a standard of 2 units of agglutinin and the highest standard was 32 units for animals tested with the first and second challenges, except for the animal No. 30 who dead and did not resist the examination of the first challenge with the standard of 8 units. Consequently, there is no possibility to rely on the serological test to ensure the animal's resistance to the disease, unless it is at high levels. Therefore, the challenge test is the criterion, since the four groups did not show a significant difference in the level of immunity during the examination of the first challenge. Nonetheless, other researcher found that the protective immuno-standard (criterion) (1/64) continues for 60 days for one dose of alum precipitated vaccine and 120 days with two doses, while the oily vaccine provides protection for 210 days with one dose compared to 300 days with two doses (Jalil *et al.,* 2010; Jaffri *et al.,* 2006). Whereas others, mentioned that the level of the immunity remains high for 270 days after vaccination with an oily vaccine, but with two doses between them for a period of two months, and this was better than the alum precipitated vaccine in the immune level (Muneer *et al.,* 1994; Tabaabaei *et al.,* 2007).

The results of this study also showed increasing in the level antibodies at day 42 or three weeks after the second dose of the vaccine for the animals in the first and second groups, which was higher than the third group vaccinated with a single dose of the oily vaccine. Though 100 % of third group animals were resisted the challenge dose. These results indicated the error or inaccuracy of serological investigations to approved the animal's resistance to the disease. However, the fact stated that the second dose was necessary for the high level of immunity for the animal vaccinated with alum precipitated vaccine, but it was not sufficient to protect 100% of animal in compare to a single dose of oily vaccine. All these results indicate the weak correlation between the level of antibodies in the animal's with the level of protection from the disease. Previous studies indicated the role of both cellular and humoral immunity against the Hemorrhagic septicemia disease (Saleem *et al.,* 2014; Benkirane and DE ALWIS, 2002). Moreover, Tabaabaei *et al.,* (2007) considered that the immune response to *Pasterulla multocida* was not well understood and protection with live vaccine may not be fully reflected in humoral immunity.

The results of this study showed that the level of antibodies in the first group increased without a significant difference after the first dose and then increased more significantly after the second dose than the other groups. Later on, decreased in the level of antibodies after 2, 4 months until approaching the lowest level in five months. While, Dagleish, (2007) was found that the level of immunity did not rise only after the second dose of vaccine.

The results of this study also revealed increasing in the body temperatures in all animals injected with the challenge dose compared to the negative control animals. While the positive control animals were died before observing the changing in body temperature. There was no significant difference in temperature or statistical significance in groups of vaccinated animals, except in few animal that were died. However, the animals in the third group reached the highest temperature (41 ° C) and decreased to (40 ° C - below), therefore, the clinical symptoms such as temperature can

be used as indicator to distinguish the animals 'resistance to the challenge dose and the efficiency of the vaccine type in immunizing buffaloes against hemorrhagic septicemia disease.

In this study, the differences between the results of antibodies level of four groups were not significant, during the examination of the second challenge because the vaccination developed animals immunity. However, local bacterial growth of the challenge dose might affected the animals or subclinical infection might occurred as in the control group. The presence of memory cells might lead to immunologically quick response without any symptoms appearing on the animals.

The results of this study also approved that all the vaccines used were highly efficient in immunizing buffalo against hemorrhagic septicemia. Moreover, vaccinated animals were resisted a high challenge in compare to the control group that all died within 24 hours after being injected with the same challenge dose.

The results of the current study also approved that the third group vaccinated with single dose of oily vaccine acquired stronger immunity than in the two groups vaccinated with two doses of alum precipitated vaccine, whether it was alone or mixed with another vaccine. Although the vaccine(HS) mixed with the (BL), vaccine has given better results than the single vaccine, it should be given in two doses as well. All these results of the vaccination program and the obstacles face the veterinarians in the field should be consider for the controlling of HS. Therefore, the use of a single dose of oily vaccine greatly reduces this problems and develop high immunity.

The vaccination protocol used in this study, in terms of method of injection, the dose and the location of vaccination and the booster dose, explains the possibilities of that led the buffalo to bypass the challenge examination for two times with a distance of six months while the disease was noticed in 2008 (Jalil *et al.,* 2010), despite the presence of the alum precipitated vaccine that used the alone and mixed with the black leg vaccine. However, the booster dose was impossible after 21 days in term of buffaloes because of the field difficulties such as the nature and behaviors of adult buffaloes, especially in the marshes. Therefore, during 2008 outbreak, the vaccine dose might not receive because of field difficulties.

In conclusion, the results of the this study approved that the single dose of oily vaccine against hemorrhagic septicemia is better in terms of immunization than alum precipitated vaccines, whether alone or mixed with other vaccines. Consequently, the authors suggested to change the buffaloes HS vaccination program in Iraq and recommend the single dose of oily vaccine instead of alum precipitated vaccine with consideration of changing in the periods of vaccination process.

References

Albaek B, Eriksen L, Rimler R. B, Leifsson P. S, Basse A, Christiansen T. and Eriksen E. (2009). Typing of *Pasteurella multocida* from haemorrhagic septicemia in Danish fallow deer (*Damadama*). APMIS. 107; 913–920.

Ali Z, Muhammad K, Hussain I. and Hameed E. (2000). Antibody response of buffaloes to hemorrhagic septicemia vaccine . Int. J. of Agri. & Biol. 3;183-186.

Ashraf A, Tariq H, Shah S. and Nadeem S.(2011). Characterization of *Pasteurella multocida* strains isolated from cattle and buffaloes in Karachi, Pakistan. African J. of Microbiol. Res. 5;(26): 4673-4677.

Bain, R.V.S., DE Alwis, M.C.L., Carter, G.R. and Gupta, B.K. (1982). Haemorrhagic Septicemia. FAO Animal Production and Health Paper. 33;FAO, Rome, Italy.

Benkirane A. and DE ALWIS M.C.L. (2002). Hemorrhagic septicemia , its significance , prevention and control in Asia . Vet. Microbiol. 41: 213-219 .

Carter G.R. (1955). A haemagglutination test for the identification of serological types. Am. J. Vet. Res. 16: 481–484.

Chandrasekaran S, Kennett L, Yeap PC, Muniandy N, Rani B. and Mukkur T. K(1994). Characterization of immune response and duration of protection in buffaloes immunized with haemorrhagic septicemia vaccines. Vet. Microbiol. 41;(3):213-9.

Dagleish M. P, Hodgson J, Ataei S, Finucane A, Finlayson J, Sales J, Parton R. and Coote J. G. (2007). Safety and protective efficacy of intramuscular vaccination with a live aroA derivative of *Pasteurella multocida* B:2 against experimental hemorrhagic septicemia in calves. Infection and immunity .American Society for Microbiology. 75;(12): 5837-5844.

DE Alwis M.C.L. (1992). Haemorrhagic septicemia – a general review. Br. Vet. J. 148; 99–112.

De Alwis M.C, Wijewardana T. G, Gomis A. I. and Vipulasiri A. A. (1990). Persistence of the carrier status in haemorrhagic septicemia (*Pasteurella multocida* serotype 6:B infection) in buffaloes. Trop. Anim. Health Prod. 22; (3):185-94.

Farooq U, Saeed Z, Khan M. A, Ali I. and Qamar M. F. (2011). Sero-surveillance of hemorrhagic septicemia in buffaloes and cattle in southern punjab Pakistan . Pakistan Veterinary Journal. 8318;(3):2074-7764 ·

Jaffri K.T, Gill Z.J, Bhatti A.R. and Raza. (2006). A. Immune response of Buffaloe calves to haemorrhagic septicemia oil adjuvant and alum precipitated vaccine . Int. J. of Agri. & Biol. 5; 645-647

Jalil Abed Gatie, Khawla Ghani and Majda Saeed. (2010). Diagnosis of haemorrhagic septicemia in buffalo marshes in southern Iraq, 2008. Al-Qadisiyah Journal of Veterinary Science. 9; 2: 62-68.

Karimkhani H, Salehi Z, Zali S, Karimkhani M. and Lameyi R. (2011). Isolation of *Pasteurella multocida* from cows and buffaloes in Urmia' s Slaughter House . Archives of Razi Institute. 66;(1): 37-41 .

Manual OF Diagnostic Tests and Vaccines. (2008). For Terrestrial Animals. 6th ed. Vol. 2 . (OIE) .739-751.

Muneer R, Akhtar S. and Afzal M. (1994). Evaluation of three oil-adjuvant vaccines against *Pasteurella multocida* in buffalo calves . Rev. Sci. tech. off. int. Epiz. 13; (3): 837-843 .

Mustafa A. A, Ghalib H.W. & Shigidi M.T. (1978). Carrier rate of *Pasteurella* herd associated with an outbreak of hemorrhagic septicemia in the Sudan. Br. Vet. J. 134: 375–378.

Naz, A., Hanif, A., Maqbool, S. and Muhammad, K. (2012). Isolation , characterization and monitoring of antibiotic resistance in *Pasteurella multocida* isolates from buffalo (*Bubalus bubalis*) herds around Lahore . The Journal of Animal and Plant Sciences. 22: 242-245 .

Priadi A. and Natalia L.(2001). Protection of a live *Pasteurella multocida* B:3.4 vaccine against haemorrhagic septicemia. J. lmu. Tern. Vet. 7; 55-61 .

Qureshi S. and Saxena H.M.(2014). Estimation of titers of antibody against *Pasteurella multocida* in cattle vaccinated with hemorrhagic septicemia alum precipitated vaccine . Veterinary World. 7; (4) : 224 – 228 .

Rosen M. N.(1981). Pasteurellosis Infectious Diseases of Wild Animals, Second Ed. Davis J.B., Karstak L.H. & Trainer D.O., eds. Iowa State University Press, Ames, Iowa, ,USA. 244–252.

Saleem, L, Munir R, Ferrari J, Afzal M. and Chaudhry F. R. (2014). Efficacy and cross – protectivity of live intranasal aerosol hemorrhagic septicemia vaccine in buffalo calves . Int. J. of curr. Microbiol. and Appl. Sci. 3; (11): 300-307 .

Sarwar N, Muhammad K, Rabbani M, Rana M, Sarwar M, Ali M.A, Nazir J. and Kamran M. (2015). Factors affecting potency of hemorrhagic septicemia vaccines . Int. J. of Agri. & Biol. 387-390 .

Shah N. H, Shah S. H. and de Graaf F. K. (1997). Protection against haemorrhagic septicemia induced by vaccination of buffalo calves with an improved oil adjuvant vaccine . FEMS Microbiology Letters. 155: 203-207

Shayegh J, Atashpaz S, Salehi T. Z. and Hejazi M. S. (2010). Potential of *Pasteurella multocida* isolated from healthy and diseased cattle and buffaloes in induction of diseases . Bull. Vet. Inst. Pulawy. 54: 299-304 .

Tabaabaei M, Moazzeni Jula G. R, Jabbari A. R. and Asmailzadeh M.(2007). Vaccine efficacy in cattle against hemorrhagic septicemia with live attenuated aroA mutant of *Pasteurella multocida* B:2 strain .Journal of Cell and Animal Biology. 1; (4):062-065.