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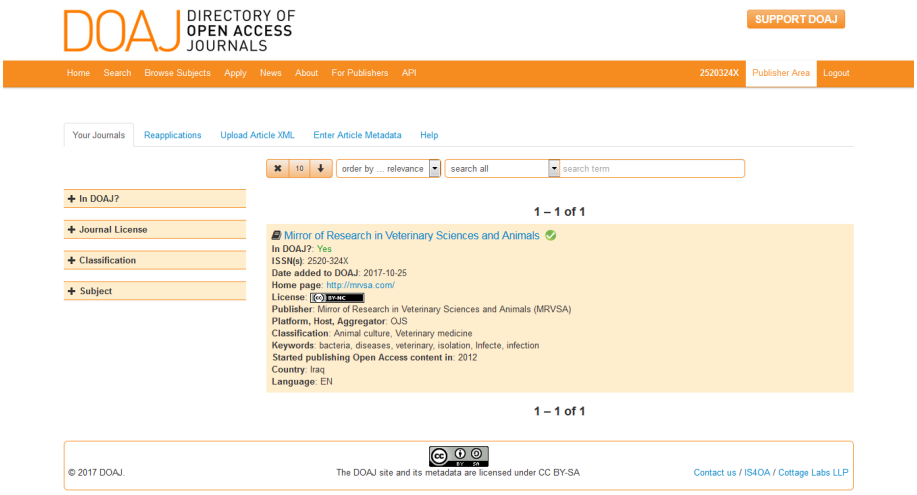

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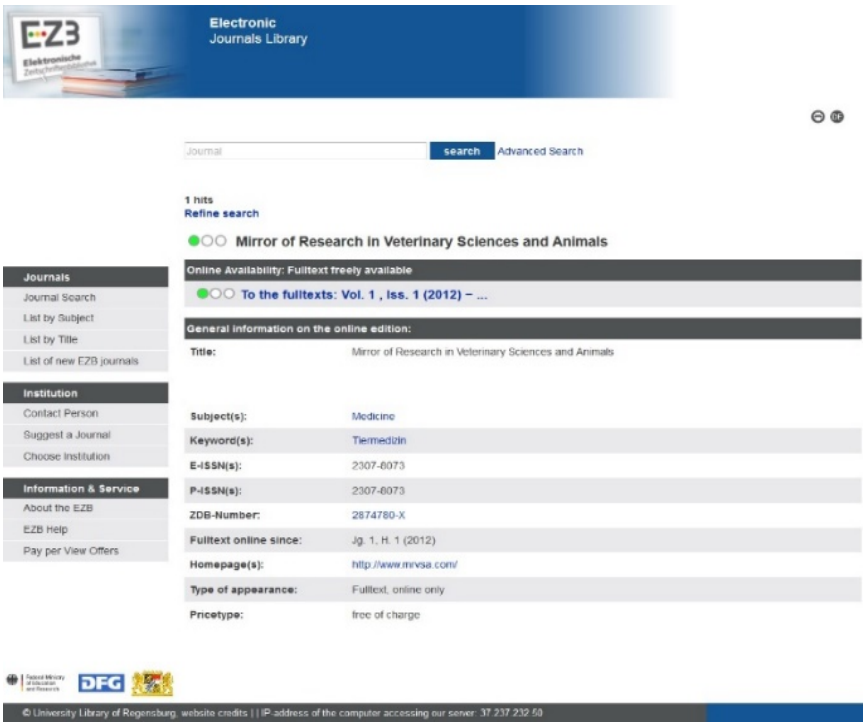

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Original ARTICLE Mirror of Research in Veterinary Sciences and Animals MRVSA/ Open Access DOAJ

First record of living fossil tadpole shrimp *Triops numidicus* (Grube, 1865 , resurrected by Naganawa) in the desert of Al-Najaf

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Abstract

The study conducted to survey the existence of tadpole shrimp in ephemeral ponds and streams of the desert of Al-Najaf. Out of 13 ponds, the tadpole shrimps were occur in 5 only including terminal part of Abu-Talah valley, archaeological basin of Um Groon castle ,first part of Wier valley, Rejlat Al-Ziana and Sharaf wells. All samples were identified morphologically as *Triops numidicus* (Grube, 1865 , resurrected by Naganawa), the controversial synonymy, morphological and morphometrical features are discussed.

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Keywords: *Triops numidicus*, desert of Al-Najaf, Notostraca, tadpole shrimps, Iraq

Introduction

Triopsidae members are freshwater crustaceans, usually called tadpole shrimp due to morphological similarity with frog larvae (Martin and Boyce, 2005). The first record of *Triops* fossil was discovered in Germany up to Devonian period, with very close morphological features to *T. cancriformis*, therefore it referred to "living fossil" (Mantovani et al., 2008). Thus, it is considered as a constant example of evolutionary stasis. *Triops* spp. especially present in warm temporary freshwater ponds particularly in arid and semiarid regions and hot climates (Rzoska, 1984; Thiéry, 1991; Williams, 2006). Although it is not yet known how these Notostraca transported between ephemeral ponds, they distributed in all continents except Antarctica (Philip, 2012). Due to plasticity of morphological features of Notostraca ,the taxonomist often describe new (Brendonck, 1996). Among two genera of Triopsidae; *Triops* and *Lepidurus*, Four species are recorded in the Middle East including *Triops canacriformis* from Yemen and Iran in addition to *Lepidurus couesii* from Syria, *Triops numidicus* from Saudi Arabia and Oman (Thiéry, 1991) , *Triops cancriformis* and *Lepidurus apuslubbocki* from Israel (Kuller and Gasith, 1996) and *Triops granarius* from Iraq (Longhurst,1955). Longhurst (1955) mentioned that the Iraqi specimens collected from saline ponds from Imara and Baghdad provinces without any more information, therefore the present study is designed to survey the desert of Al-Najaf to reveal occurrence of Notostraca in this expected suitable habitats .

Material and Methods

Study area The Najaf desert is an extension of the northern plateau of the Arabian Peninsula and is part of the southern desert of Iraq .In general, the Najaf desert area is flat with an obvious rise towards the south, reaching its highest height near the Iraqi-Saudi border (300-400msl). The majority of the landscape is a plateau that is dissected by a large number of valleys of different lengths, which are drained rain water towards the north eastern part. Depressions are a distinctive feature of the Najaf desert, and they are either the result of erosional or solutional (krast), their sizes and shapes differ. They are circular, oval, or longitudinal, and some of them collect rain water to be green meadow in spring season called locally Faidah , another may hold rain water for several months, which forms a large water body in addition to some ephemeral water ponds occur due to water stagnation in base of some ephemeral streams (Ma'ala, 2009).

Sampling

During the rainy season in March 2020, the presence of tadpole shrimps in the temporary ponds was investigated, as 13 ponds were examined in different places of the Najaf desert .The presence of tadpole revealed by careful observation to detect either tadpole shrimp swimming or dead bodies on the edge of the basin, sometimes and during night visits the spotlight is highlighted on the basin to attract the swimming individuals. Samples were collected from the habitat using plankton net and immediately transferred to container with absolute ethanol. Specimens were identified using appropriate taxonomic references (Longhurst, 1955; Alonso, 1985, 1996; Kuller and Gasith, 1996). Fifty individuals were examined for morphometrical characteristics using a stereomicroscope.

Results and discussion

Occurrence of tadpole shrimp

The tadpole shrimp are reported in temporary ponds distributed in 5 areas (map1) including:

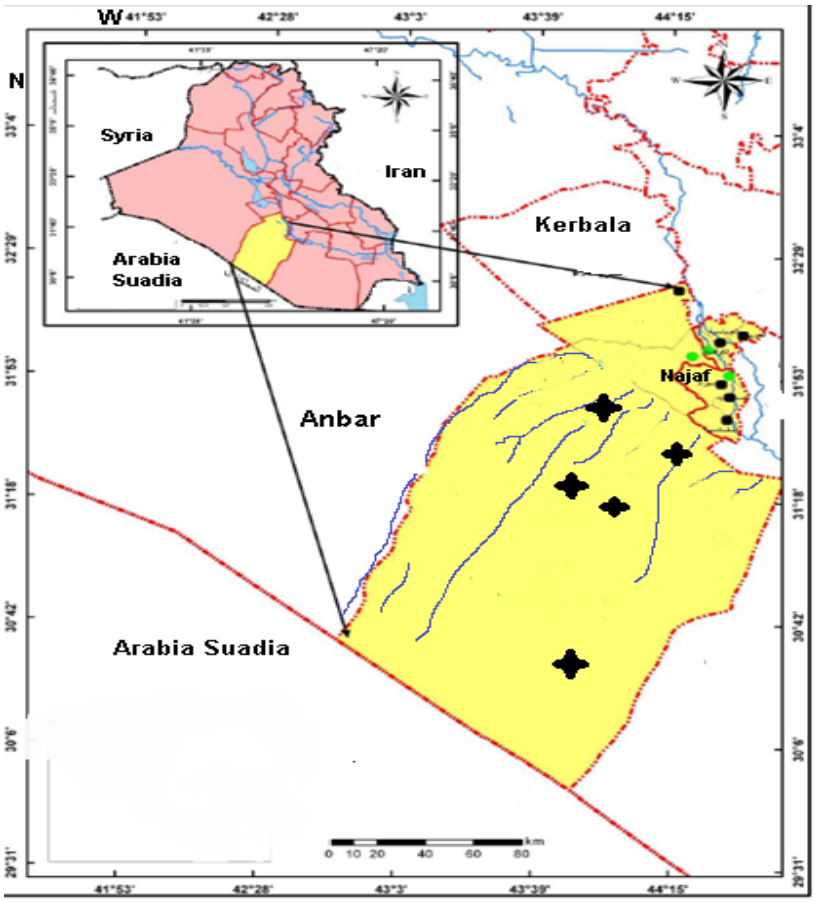
Site 1: (figure 1, A) terminal part of Abu- Talh valley (31.660497 N,44.309093E). It is a small temporary pond about 6 ×2.5 m with 0.62m depth. The bottom of pond covered with flat rocks with muddy edges. This pond was crowded with alive tadpole shrimps with many dead bodies around it. The salinity and pH of water were 0.03% and 8.27 respectively.

Site 2: It is a part of an archaeological cistern (31.537703N, 44.229974E) dedicated to collecting rain water. The surface area of the basin is about 2000m^2 and with a maximum depth of 70cm, the floor of basin covered with thick clay layer. The salinity and pH of water were 0.01% and 8.04 respectively.

Site 3: first part of Wier valley (31.61941N,44.226857). It is a small temporary shallow pond of 0.7m depth measured 7 ×0.75 m. The pond was crowded with alive tadpole shrimps with many dead bodies at edges. The salinity and pH of water were 0.1% and 8.77 respectively.

Site 4: Artificially engraved large pond at Rejlat Al-Ziana (31.625125N,43.985908E) about 40 × 8m. The tadpole shrimps was observed only at night by attracting them by spot light. The salinity and pH of water were 0.1% and 7.7 respectively..

Site 5: Sharaf wells: They are a large group of holes of 1-1.5m diameter engraved in a rocky layer dedicated to collecting rain water (30.621343N,43.741711E). Most of these wells are filled with mud except two, both of which contain tadpole shrimps. The salinity and pH of water were 0.09% and 8.1 respectively.



Map 1: the study area at the desert of Al-Najaf, southwestern Iraq

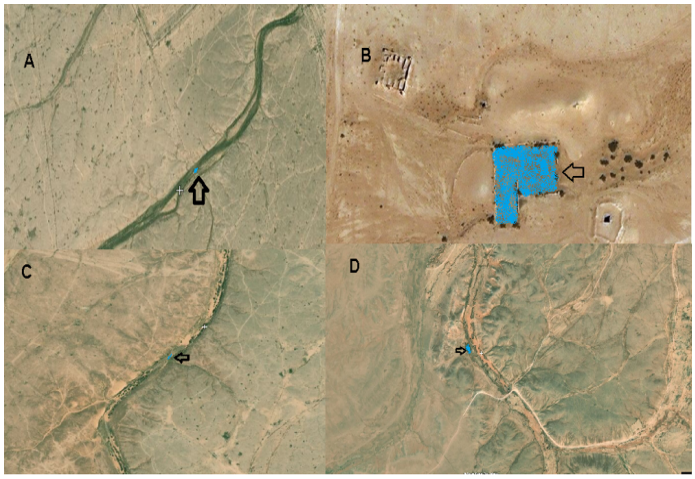


Figure 1: sites of ephemeral ponds contain tadpole shrimps, A: Abu Talah valley: B: Um Groon castle basin ; C: Wier valley ; D: Rejlat Al-Ziana

All sites mentioned above are suitable to live and reproduction of tadpole shrimps, Pennak (1978) indicated that the characteristic habitat of Notostracans consists of muddy, alkaline pools which dry completely in the warm months. This type of intermittent habitat is usually found in the desert of Al-Najaf. Tadpole shrimp is one of the most important animals in ephemeral wetlands and even the surrounding landscape, where they strongly affect faunal structure (Yee et al., 2005). The genus *Triops* occurs in temporary wetlands in arid areas worldwide, spanning periods of drought by laying desiccation-resistant eggs (or cysts) that can lay dormant and viable for years in unflooded soil (Brendonck and de Meester, 2003; Brendonck et al., 2008). Soon after re-immersion in water, a portion of the eggs hatch, and within a few weeks adult tadpole shrimp deposit hundreds to thousands of eggs before the ephemeral water-bodies dry again (Brendonck, 1996). It is not yet known how the tadpole moves between the temporary basins in the arid desert, although some researchers have indicated that it is likely to be transported by waterfowl, car's tires, and boots (Brochet et al., 2010;Waterkeyn et al., 2010). In the present study, the sites 1-4 are connected to a temporary watercourse network where rainwater runs during the flood season.

Identification: all samples are identified as following:
Family Triopsidae Keilhack, 1909
Genus *Triops* Schrank, 1803
Triops numidicus (Grube, 1865), resurrected by Naganawa

Synonyms

The scientific designation and its synonyms are still controversial and it summarized as following:
non *Apus granarius* Lucas, 1864
non *Apus numidicus* var. *dybowskii* Braem, 1893
non *Apus numidicus* in Uéno (1925)
non *Apus numidicus* var. *sinensis* Uéno, 1925

non Triops granarius in Longhurst, 1955 [in part]
non *Apus dispar* in Forró and Brtek (1984)
non *Triops granaries dispar* in Forró and Brtek (1984)
= *Apus numidicus* Grube, 1865
= *Apus dispar* Brauer, 1877
= *Apus dukianus* Day, 1880
= *Apus numidicus* in Simon (1886) [in part]
= *Apus numidicus* var. *strauchii* Braem, 1893
= *Apus somalicus* Wedenissow, 1895
= *Apus bottegoi* Del Prato, 1896
= *Apus numidicus* in G. O. Sars (1898)
= *Apus bottegoi* in Bouvier (1899) [non 1898]
= *Apus numidicus* in G. O. Sars (1899)
= *Apus trachyaspis* G. O. Sars, 1899
= *Apus numidicus* in G. O. Sars (1905)
= *Apus numidicus* in Stebbing (1910)
= *Apus trachyaspis* Stebbing (1910)
= *Apus zanoni* Colosi, 1920
= *Proterothriops zanoni* in Ghigi (1921)
= *Proterothriops somalicus* in Colosi (1923)
= *Apus numidicus* in Barnard (1924) [in part]
= *Apus numidicus* in Gurney (1924)
= *Apus numidicus* in Pérès (1939)
= *Triops* cf. *granarius* (sp.3) from Tunisia in Naganawa (2018)

Synonymy complex; however, in the light of current scientific standards, Barnard's (1929) synonymy is the most reliable one. Since Longhurst's (1955) revision, this species has been confused under the name of "*Triops granarius*" for many years, e.g., *Triops numidicus* and *Triops granaries* s.str. are being used for the same populations by different authors. These are, however, genetically independent each other (Naganawa, 2018). *Triops numidicus* is the species originated from Africa; whereas *Triops granaries* s.str. (Redefined by Naganawa) is the species distributing in East Asia only. The former is by far the commonest and the most widely distributed *Triops* species throughout Africa, and partly reaches over the Arabian Peninsula, but not confirmed in more east than Afghanistan, therefore, Naganawa (2018) suggests herein the binomen "*Triops granarius*" be restricted to the population in East Asia. This suggestion is not an option under the International Code of Zoological Nomenclature. Thus, Naganawa (2018) proposes resurrecting this species name of *Triops numidicus*.

Types

None designated in the original description (Grube, 1865). Naganawa (2018) confirmed that no type of this species is deposited at the Natural History Museum Vienna, Austria; but the type of *Apus dispar* Brauer, 1877 is deposited there. [cf. Type of *Apus trachyaspis* G. O. Sars, 1899 is deposited in the South African Museum, Cape Town, South Africa.]

Diagnosis

Dorsal organ depressed and (sub) triangular or trapezoidal. Carapace oval, its length (including the posterior angles) a little greater than its width, more or less arched, usually convex along whole lateral margin to posterior angle. About 30, varying 25-33 in both sexes (figure 2), abdominal segments are uncovered by carapace. Number of apodal (legless) segments normally 13-14 (males) or 10 (females), varying 12-15 (males) or 9-13 (females). Carina lacks spines. Sulcus cut not very deep, on the concave margin about 20-34 denticles (almost small rounded protuberances) on each side (42-63 in total). Fourth endite of 1st leg usually as long as, or a little longer than carapace. Males having strong scales on furcal filaments (caudal rami) as Asian *Triops spp*. Furcal filaments in females are so long, but in males about as long as carapace (including posterior angles) ,figure 2.

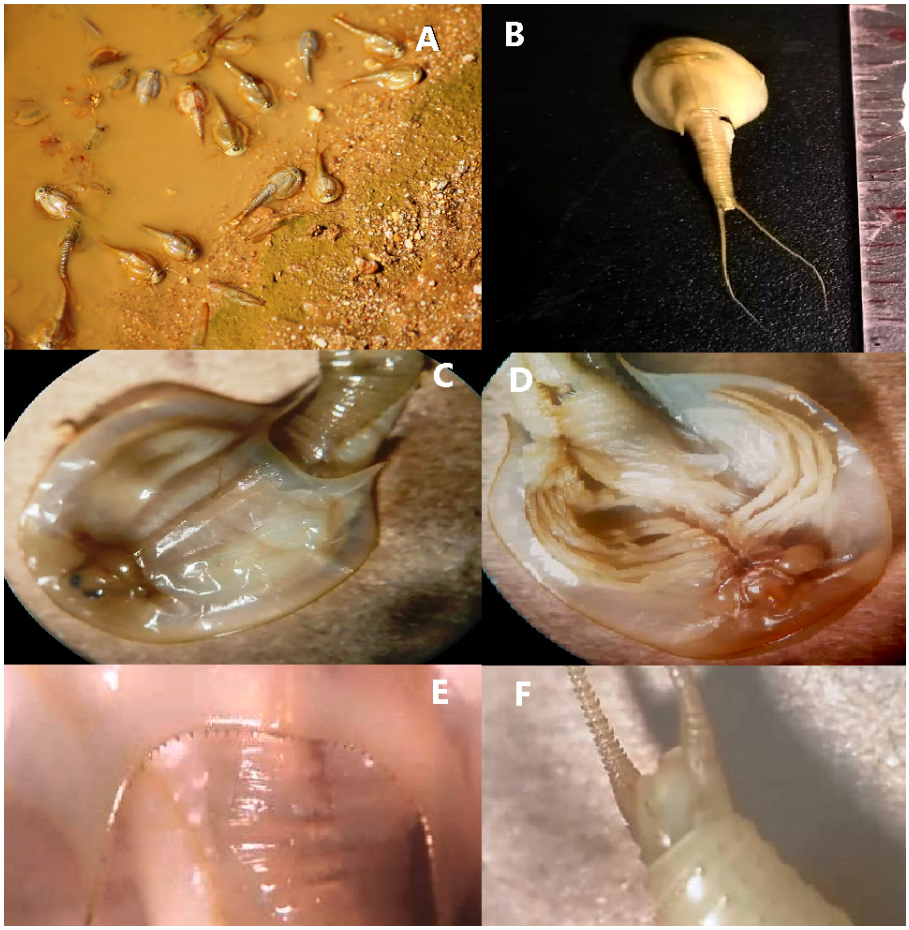


Figure 2: Shows A: *Triops numidicus* swimming in temporary pond at Wier valley ;B: general appearance of Triops numidicus; C: dorsal view of Carapace ;D: ventral view of Carapace ;E: posterior margin carapace teeth ; F: dorsal view of telson.

Distribution: North Africa (Sahara Desert: Algeria, Tunisia, Libya, Sudan), East Africa (Somalia, Kenya), South Africa (Namibia, Botswana, South Africa), Arabia, Iraq, Afghanistan.

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Paralytic Cattle Syndrome: causes and treatments in Mali

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Abstract

A study was conducted between 1999 and 2005 to understand cattle paralytic syndrome causes, which are of great concerns for breeders in the Sahelian and sub humid areas of Mali and to find ways of combating it. Survey was carried out in the regions of Kayes, Koulikoro, Sikasso, Timbuktu and Gao, where 29 samples of fodder and 415 of blood were taken. The plasma was extracted by centrifugation at 3000 rpm for 10 minutes. Dry matter, crude protein, organic matter, cellulose, phosphorus, calcium, potassium, magnesium, sodium, zinc, copper, manganese and iron were determinate in the fodder, and total protein, albumin, total globulin, Alpha, Beta and Gamma globulin, globulin / albumin ratio, calcium, phosphorus, magnesium, zinc and copper in the plasma samples. Then, a treatment trial was conducted in three areas with veterinary drugs: calcimag, hipracal-FM, and cofacalcium at doses of 5 ml / 10 kg bodyweight at one day apart and 1 ml / kg bodyweight three days apart. Fodder had low levels of crude protein (4.5 ± 0.62%), calcium (0.3 ± 0.03%), phosphorus (0.1 ± 0.01%) and magnesium (0.3 ± 0.02%). Biochemical parameters were statistically the same in all animals regardless of the clinical state with the exception of calcium, which was lower (2.25 mmol / l) in patients versus 2.67 mmol/l in the healthy ($p = 0.028$). Animals treated with the licking stone were 100% healed, while with injectable drugs, the healing rate varied between 73% and 86%, compared to 1% in the control group. The cost of treatment varied between 5.72 and 2.42 US dollars depending on drug and doses. These results are being used by extension services in the study areas.

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Keywords: Causes, chemical composition, plasma biochemical profile, cure rate.

Résumé

Une étude a été conduite entre 1999 et 2005 pour comprendre les causes du syndrome paralytique des bovins qui constitue une préoccupation des éleveurs des zones sahélienne et subhumide du Mali et d'en trouver les moyens de lutte. Une enquête a été effectuée dans les régions de Kayes, Koulikoro, Sikasso, Tombouctou et de Gao, au cours de laquelle 29 échantillons de fourrages et 415 de sang ont été pris. Ensuite, le plasma a été extrait par centrifugation à 3000 tours/minute pendant 10 minutes. La matière sèche, les protéines brutes, la matière organique, la cellulose, le phosphore, le calcium, le potassium, le magnésium, le sodium, le zinc, le cuivre, le manganèse et le fer ont été déterminés dans les échantillons de fourrages, tandis que la protéine totale, l'albumine, la globuline totale, Alpha, Béta et Gamma globuline, le rapport globuline/albumine, le calcium, le phosphore, le magnésium, le zinc et le cuivre ont analysés dans le plasma. Ensuite, un essai de traitement a été conduit dans trois localités avec des produits vétérinaires : calcimag, hipracal-FM, et cofacalcium aux doses de 5 ml/10 kg de poids vif à un jour d'intervalle et 1 ml/kg de poids vif à trois jours d'intervalle. Les fourrages avaient des teneurs faibles en protéines brutes (4,5 ± 0,62%), en calcium (0,3 ± 0,03%), en phosphore (0,1 ± 0,01 %) et en magnésium (0,3 ± 0,02%). Les paramètres biochimiques étaient statistiquement les mêmes chez tous les animaux quel que soit l'état clinique à l'exception du calcium, qui était plus bas (2,25 mmol/l) chez les malades contre 2,67 mmol/l chez les sains (p = 0,028). Les animaux traités avec la pierre à lécher ont été guéris à 100%, tandis qu'avec les produits injectables, le taux de guérison a varié entre 73 % et 86%, contre 1% dans le lot témoin. Le coût du traitement a varié entre 5,72 et 2,42 dollars Etats-Unis en fonction dal quantité des produits et des doses. Ces résultats sont en cours d'utilisation par les services de vulgarisation dans les zones d'étude.

Mots clés : Causes, composition chimique, profil biochimique plasmatique, taux guérison

Introduction

Since some thirty years, breeders in the Sahelian zone of Mali have been confronted with a disease called "differently" according to ethnic groups: "Dissidimi bana" in Bambara, "Bougueinshi" in Moor, "Moubraïka" in Tamacheck (Traoré, 1985; Kouyaté, 1988), Gountè in sonhrai in the Tonka area, Bardi in Moor in the Tinhara and Gossi area, Tadacart dacard which means staggering disease in Tamacheck in the Anderamboukane area (Ouologuem *et al.,* 2006b). Once seasonal and isolated, this disease has now become endemic in many localities. The geographic area of the disease covers a South-North zone from the beginning of the Sahel to Mauritania and an East-West zone from Tilemsi to the Senegalese border (Kouyaté, 1988). However, several cases have been encountered in southern Mali where rainfall is between 900 and 1,200 mm / year (Ouologuem *et al.,* 2006a).

In each herd, the rate of disease affected animals varies between 3 and 5% (Ouologuem *et al.,* 2006a). Cattle aged 3 years and older and in good condition are the main target of

the disease. The main features of the syndrome are: pica, including osteophagia, motor incoordination, decubitus, rapid death or slow healing (Traoré, 1985; Kouyaté 1988; Ouologuem *et al.,* 1998 and 1999).
Clinical signs recall the pathology which had raged in the 1965s in the Ferlo in Senegal called "Gniédio" in fulani and described by many researchers (Calvet *et al.,* 1965; Calvet,1971; Calvet *et al.,* 1976; Friot *et al.,* 1971; Friot *et al.,* 1973; Conrad *et al.,* 1985).
In researching this pathology cause, Calvet *et al.,* (1965) isolated *Clostridium botulinus* serotypes C and D from dead animals tissues with all the clinical signs. They have not only been able to reproduce the disease by administering parts of organs from suspect animals to healthy ones, but also to limit the mortality of healthy animals by vaccination with the vaccine prepared from the two serotypes. According to these authors, if mineral deficiency, in particular phosphorus is the cause of osteophagy, the high mortality of animals and which is often of an abrupt nature, is due to botulinum toxins. Conrad *et al.,* (1985) reported that in several Latin American countries the appearance of botulism is linked to the existence of phosphorus deficiency and osteophagy.
However in Mali, treatments with veterinary products (antibiotics, anthelmintics, vaccinations in particular against botulism) have not given satisfactory results (Kouyaté, 1988), although a deficiency in phosphorus, copper and selenium has been observed in serum analyzes by Kouyaté *et al.,* (1995). On the other hand, all botulinum toxin analyzes results were negative (Ouologuem *et al.,* 2001) as the attempts to contaminate healthy animals from sick animal organs did not cause the disease. The objective of this study was to deepen the knowledge in the nutritional aspect as probable cause of the patient and to propose a treatment.

Materials and methods

Test 1: Diagnosis of pathology causes
Surveys were carried out in Kayes, Koulikoro, Sikasso, Timbuktu and Gao regions. During this survey, 29 samples of pasture fodder and 415 blood samples were taken. Three groups of animals were identified during the collection time: those apparently healthy, those showing syndrome signs and those having suffered from the pathology in the past according to the breeders.
Animals blood samples were taken early in the morning before going for grazing. Plasma was obtained immediately after collection by centrifugation at 3000 rpm for 10 minutes. Then, samples were kept cold at 4 ° C in the field and kept at - 20 ° C in the laboratory before sending them for analysis to the Inter-State School of Veterinary Sciences and Medicine biochemistry laboratory (EISMV) of Dakar (Senegal) for the following determinations: total proteins, albumin, α, β, γ globulin, Ca, P, Mg, Zn, Cu. The plasma proteins were analyzed by spectrophotometry after the Biuret reaction at 545 nm. Then, protein fractions were analyzed by electrophoresis and read using a densitometer. Minerals were measured spectrophotometrically at different wavelengths depending on the elements.
In some localities, supine patients were sacrificed and autopsies were performed, during which special attention was paid to the cartilages joints state of the forelimbs and hindquarters state.
Laboratory data were analyzed through descriptive statistics (frequency, arithmetic mean, coefficient of variation) and variance by considering the clinical condition of the animals as a factor. The means were compared by the orthogonal contrast method.

Test 2. Patient treatments

A treatment experiment on patients was conducted in Sikasso (southern Mali), in Timbuktu and Gao (northern Mali), regions. As sick animals and their clinical statuses were identified, they were divided into four batches.
Batch 1 (check): basic ration (unlimited bush straw + 1 kg of Huicoma cattle feed - ABH). ABH contained 0.46 UF of net energy and 139 g of digestible nitrogenous matter (MAD) per kilogram of dry matter, while bush straw contained 0.40 UF and zero grams of MAD.
Batch 2: injection of 5 ml /10 kg bodyweight of Cofacalcium or hipracal-FM, or CalciMag (veterinary solutions containing calcium, phosphorus and magnesium) one day apart + basic ration (unlimited straw + 1 kg ABH);
Batch 3: injection of 1 ml / kg bodyweight of Cofacalcium or hipracal-FM or CalciMag three days apart + basic ration (unlimited straw + 1 kg of ABH);
Botch 4: animal lick stone (photo 1) + basic ration (unlimited straw + 1 kg of ABH)
Batches 2 and 3 were each subdivided into three subgroups where each received an injectable product. Batch 1, 2 and 3 consisted decubitus animals or very difficult to move, while those of batch 4 moved more easily. Products used were administered by intravenous injection. The dose used in batch 1 was that indicated on the instructions for use, while that in batch 2 was proposed by the research team. Treatment continued until the animals showed no signs of the disease or died despite treatment. Animals have not undergone any other treatment such as antiparasitics, anti-inflammatories, antibiotics or others.

Ethical & research approval

During the implementation of this theme, there was no Ethics Committee at the "Comité National de la Recherche Agronomique-CNRA level in Mali. But, the theme on "Paralytic Cattle Syndrome: Causes and Treatments in Mali" has been included in the Malian Institut d'Economie Rurale-IER research program since 1995. The research protocol was accepted during the 2nd session of the IER Committee Program. On the basis of results obtained between 1995 and 2002, the Regional Commissions of Research Result Users (CRU) of Sikasso and Gao regions requested treatment experimentations in their respective regions through call N ° 006 / 04 / RD / CRRVA-GAO of the National Committee for Agricultural Research-NCAR.
The treatment protocols of the Institute d'Economie Rural of 2004 were validated by the CRRVA and NCAR. From an ethical standpoint, all cattle owners have been informed of all handlings their animal should undergo. After these awareness activities, cattle owners freely joined the program. It was cattle owners who phoned the research staff for the presence of sick animals in their herds and monitored treatment progress. All reports were presented to the CRA, CRRVA and CNRA. After validation, feedback sessions were organized to share results in both regions. This is why cattle treatments are applied nationwide

Results

Test 1: Diagnosis of the pathology causes ±

Chemical composition of range fodder

Fodder on the pastures used by cattle herds was very poor in protein, calcium, phosphorus and magnesium, while microelements contents in particular of iron and manganese were very high (Table 1).

Table 1: organic and mineral content of fodder sampled in pasture areas in 2002 in the region of Kayes (content/100)

Variables	N	Mean	Minimum	Maximum
Total nitrogen matter	29	4.50±0.62	1.06	13.06
Ash	29	5.60±0.48	2.35	12.80
Cellulose	29	40.14±1.30	22.79	49.00
Sodium	29	0.35±0.09	0.05	1.67
Phosphorus	29	0.10±0.01	trace	0.25
Calcium	29	0.30±0.03	trace	0.86
Potassium	29	1.40±0.09	0.33	2.29
Magnesium	29	0.23±0.02	0.1	0.49
Zinc, mg/kg	29	19.83±1.77	2.6	48.70
Copper, mg/kg	29	11.45±1.19	trace	20.83
Manganese, mg/kg	29	197.50±43.50	48.8	1027.60
Iron, mg/kg	29	258.80±57.60	10.1	1055.50

Biochemical parameters of the serum based on animal clinical states

Calcium was the only element that was different between healthy and healed animals on the one hand and sick ones on the other hand (Table 2). All other parameters were statistically the same between the three clinical states of the animals.

Findings after sacrificed animals' autopsies

Foreign bodies were found in all sacrificed animals rumen, while internal organs were apparently normal. However, cartilage erosion has been observed in the scapular - humeral, humero - radio-ulnar or femuro-tubio-patellar joints of all animals. This erosion was superficial on some joints (Photo 1), but on others, it was deep, causing a bone sore, especially in animals in decubitus (Photo 2).

Test 2. Treatment test for sick animals

Patients and control results animal treatments with various products are presented in Table 3. Four days after treatment onset, an improvement was noted in batches 2, 3 and 4, while in control batch, mortalities were recorded. After eight days of treatment, disease external signs disappearance was noted in certain patients in batches 2, 3 and 4 who started a normal walk and were eating properly, while mortality continued in the control batch. The first cases of mortality were observed in batches 2 and 3. On the other hand, in batch 4, no mortality was recorded. The relatively high mortality rate in batches 2 and 3 is largely explained by the shepherds leaving sick animals in the pastures or in the herds departure camps for transhumant.

Table 2: proteins, albumin, globulin fractions and minerals contents according to the clinical state of surveyed animals in Kayes region, 2002.

Serum parameters	«Healthy» (n= 393)	« healed» (n = 9)	« sicks » (n = 14)	Mean±SE (n = 415)	*P*
Protein, g/l	71.1	81.6	74.2	71.3±0.69	0.247
Albumin, g/l	32.2	40.4	33.7	32.9±0.43	0.206
Globulin, g/l	35.9	37.5	36.4	35.9±0.51	0.959
αglobulin, g/l	8.2	10.4	10	8.2±0.12	0.169
βglobulin, g/l	6.2	8.8	6.6	6.3±0.16	0.28
γ globulin, g/l	22.1	18	21.8	22.1±0.35	0.506
Albumin/Globulin	0.93	1.07	0.95	0.93±0.01	0.541
Mg, mmol/l	1.77a	1.20a	1.07a	1.74±0.36	0.937
Ca, mmol/l	**2.67^{a}**	**2.59^{ab}**	**2.25^{b}**	**2.66**±0.03	**0.028**
P, mmol/l	1.65	1.38	1.68	1.65±0.03	0.646
Zn, µmol/l	2.82	2.8	3.37	2.84±0.05	0.232
Cu, µmol/l	2.69	2.18	2.49	2.68±0.06	0.619

On the bold line, numbers followed by the same letter are not statistically different at the 5% threshold.

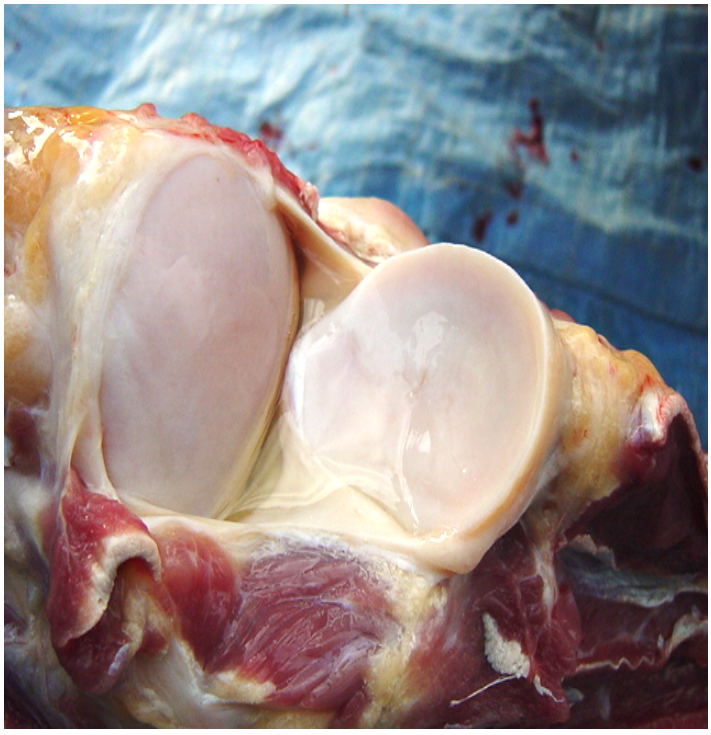


Photo 1: Superficial erosion of the humero-radio-ulnar joint cartilage of cattle in the région of Kayes in 2002

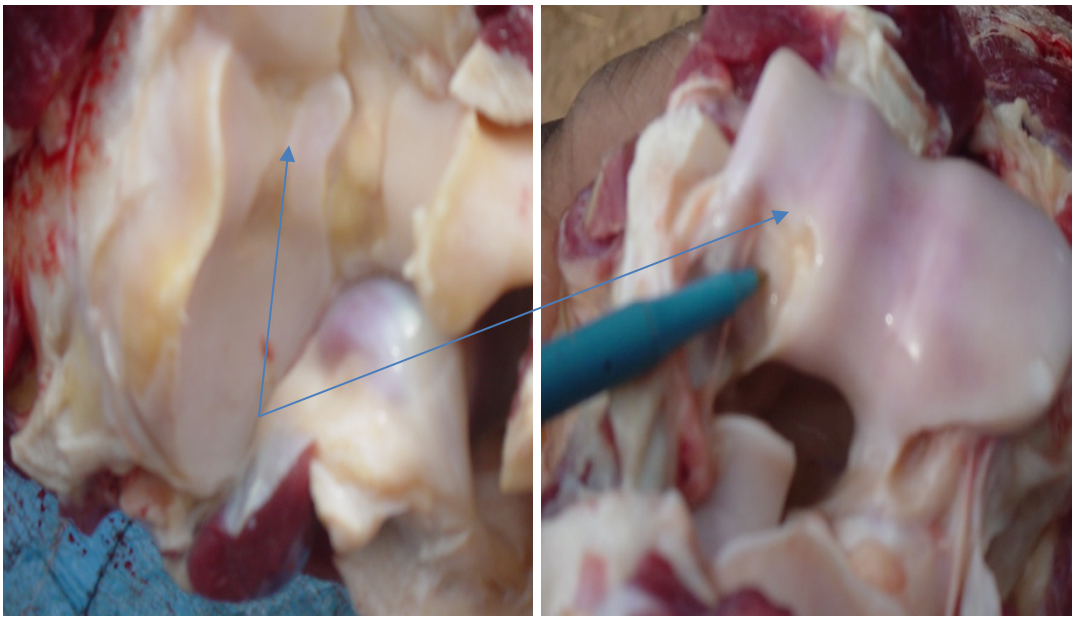


Photo 2: Deep erosion of the forelimb joints cartilage of cattle in the region of Kayes in 2002.

Table 3: Balance of treatments in different batches based on number of days and clinical conditions of animals in 2005 – 2007

Number of days of treatment	Clinical conditions	Batch 1 N	%	Batch 2 N	%	Batch 3 N	%	Batc 4 N	%
0	Decubitus	10	45.5	23	36.5	14	24.1	0	0
	Difficulty of movement	12	54.5	40	63.5	44	75.9	6	100
	Total	**22**	**100.0**	**63**	**100**	**58**	**100**	**6**	**100**
4	Worsening	8	36.4	3	4.7	3	5.2	0	0
	Improvement	0		42	66.7	43	74.1	6	100
	Dead	4	18.2	0	0	0	0	0	0
	No improvement	10	45.5	18	28.6	12	20.7	0	0
	Total	**22**	**100.0**	**63**	**100**	**58**	**100**	**6**	**100**
8	Slaughtered *	2	9.1	1	1.6	1	1.7	0	0
	Worsening	9	40.9	1	1.6	0	0	0	0
	Improvement	0	0.0	26	41.3	27	46.6	4	66.7
	Healed	0	0.0	20	31.7	24	41.4	2	33.3
	Dead	2	9.1	12	19	5	8.6	0	0
	No improvement	9	40.9	3	4.8	1	1.7	0	0
	Total	**22**	**100.0**	**63**	**100**	**5**	**100**	**0**	**100**
12	Slaughtered	4	18.2	1	1.6	2	3.5	0	0
	Improvement	2	9.1	12	19.1	7	12.1	0	0
	Healed	0	0.0	36	57.1	43	74.1	6	100
	Dead	16	72.7	14	22.2	6	10.3	0	0
	Total	**22**	**100.0**	**63**	**100**	**58**	**100**	**6**	**100**
	Slaughtered	4	18.2	1	1.6	2	3.5	0	0

20	Healed	1	4.5	46	73	50	86.2	6	100
	Dead	16	72.7	16	25.4	6	10.3	0	0
	Improvement	1	4.5	0	0	0	0	0	0
	Total	**22**	**100.0**	**63**	**100**	**58**	**100**	**6**	**100**
Treatment cost/animal (F CFA)		0		2980 (315)		3446 (290)		1750 (0)	
Treatment cost in $USD		0		4.95 (0.5)		5.72 (0.5)		2.42 (0.5)	

*** Owners sacrificed some patients in extremis without notifying treatment team. Numbers in parentheses indicate standard errors**

In addition, owners killed some patients without notifying the experiment monitoring officer. After 20 days of treatment, healing rate was very high in batches 2, 3 and 4 having received treatments, compared to high patients' mortality in the control batch.
The cost of treatment per animal was relatively low and varied between 1,750 F CFA and 3,446 F CFA or 2.42 and 5.72 $ US depending on the amount of drug used.
The three products used were effective in treating the disease (graph 1). Healing rate obtained with the different doses of calcimag was similar, while for cofacalcium and hipracal, this rate was higher with 1 ml / kg bodyweight dose of animals every 4 days compared with 5 ml / 10 kg bodyweight dose every two days as indicated on the label.

Discussion

Fodder composition

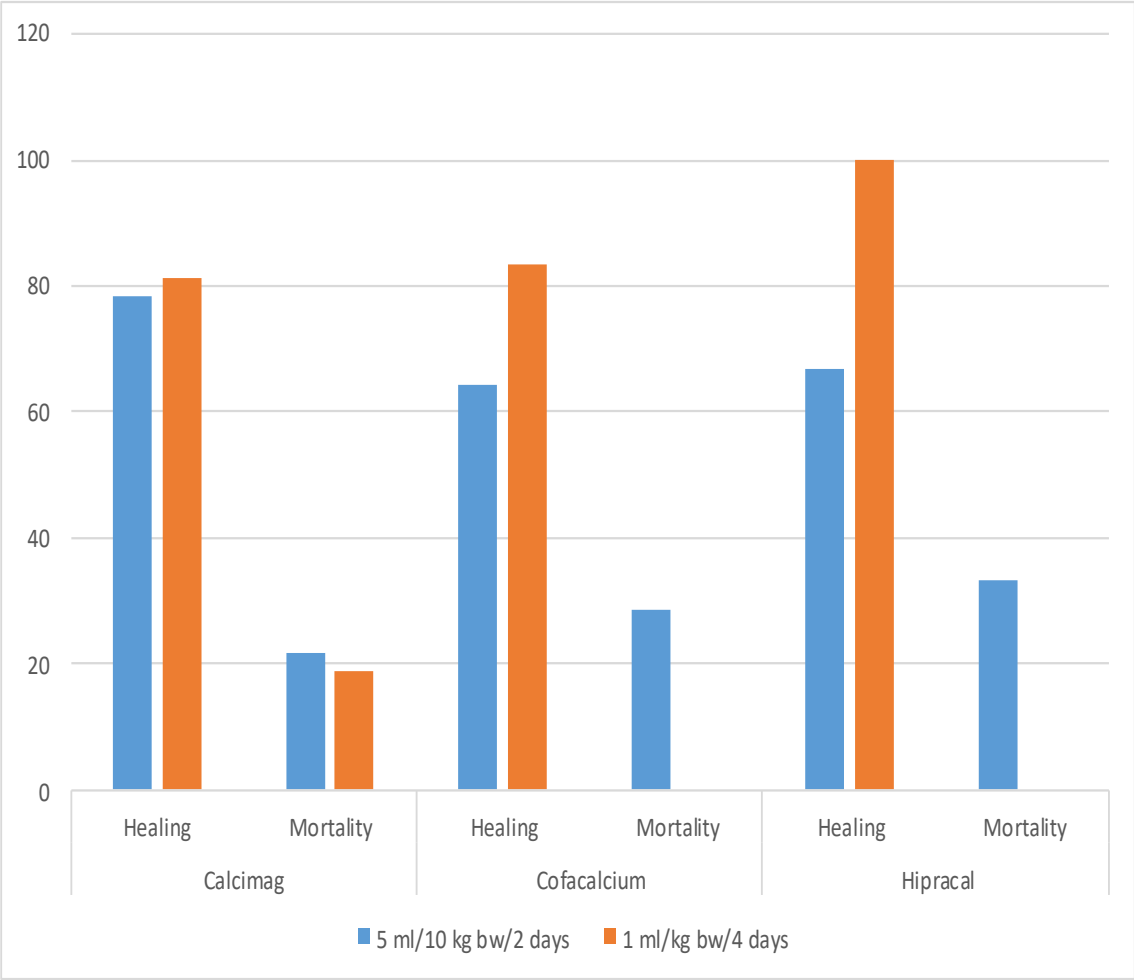
Average fodder protein content was 4.5% (1.06 - 13.06%). This low rate of total nitrogenous matter in the fodder of natural rangelands confirms previous studies conducted in the Sahelian zone such as Lambourne et al (1983) whose results varied between 3.7% and 23.1%. However, despite this low rate, animals do select feed whose ingested portion contained a crude protein level varying between 7.6% and 23.6%. Penning de Vries and Djiteye (1991) reported that maximum use of natural range biomass is achieved when the nitrogen level is 1.5% or 9.34% crude protein. Therefore, animals from the study area must make a strong species selection to gain an optimal level of protein. Dicko (1980) has reported that grazing time is inversely related to fodder availability. In addition, Lambourne et al. (1983) reported that under traditional herd management conditions in the Sahel, the need for energy maintenance increases by 42%, while quantity of ingested dry matter is strongly correlated (r =0.89) with the level of crude protein in feed (Dicko, 1980; Lambourne et al., 1983). Furthermore, Demarquilly and Weiss (1970) indicated that the digestible protein content becomes zero when the crude protein content drops to 3.8% or less than 0.6% nitrogen. Therefore, given all this information and the protein level found in the feed, it is obvious that animals in the study area were in a difficult nutritional situation.

Biochemical profile of plasma

Total proteins

The proteinemia varied between 71.1 and 81.6 g / l with an average of 71.3 g/l. The absence of a significant difference between clinically healthy animals on the one hand, healed animals, and patients on the other hand indicate that this variable cannot be taken as a criterion in the disease differential diagnosis. The interval obtained here is comparable to that of Petit and Queval (1973) in Chad which varied between 71.97 and 74.36 g / l.

Blancou et al. (1974) found a value that varied between 80 and 90 g / l in Madagascar, which was higher than the present results. These are also lower than the 81 - 86 g / l (Sawadogo et al. 1991) and the 78 g / l (Sawadogo et al. 1993) in Senegal. Boudergues and Calvet (1971) found 88.2 g / l in January and 72.7 g / l in July. July finding is comparable to the present results, but that of January is higher. Labouche (1964) indicated that total serum proteins content of tropical animals varied between 64.5 and 89.2 g / l, while indicating that several factors such as season (feeding conditions), gestation, breed, age may have influences. However, Abouna (1990) did not observe any statistical difference between non-weaned calves (83 ± 10 g / l), weaned calves (84 ± 7 g / l) and adults (87 ± 9 g / l) among the Goudali and Choa breeds of Cameroon. The fact that our results are lower than those of these authors indicates the probable existence of a nutritional problem in the current study area.



Graph 1: Results of treatments of animals with calcimag, cofacalium and hypracal at two doses

The albumin content which varied between 32.2 and 40.4 g / l is comparable to 33 g / l of Sawadogo et al. (1993), and to 34.89 g / l of Petit and Queval (1973) and to 39 g / l (Sawadogo et al.,1991).
Globulin concentrations varied between 35.9 and 37.5 g / l. Labouche (1964) reported globulin concentration norm in Senegalese cattle varied between 50 and 55 g / l, which is

significantly higher than the present results. Globulin low concentrations may be due to area animals under nutrition or to other factors needing clarification.
The alpha globulin concentration fluctuated between 8.2 and 10.4 g / l. These values are comparable to 9 g / l (Sawadogo,1993), but are lower than the 12.2 g / l (Sawadogo et al., 1991), and 14.78 g / l (Petit and Quéval, 1973). The beta globulin content varied between 6.2 and 8.8 g / l, which is lower than the 22.63 g / l (Petit and Quéval,1973) and the 16 g / l (Sawadogo et al.,1991) , but comparable to 8 g / l (Sawadogo et al,1993).
The gamma globulin fraction varied between 18.0 and 21.8 g / l. These values are lower than those of Sawadogo et al. (1991) which was 25 ± 6 g / l, 28 ± 4 g / l (Sawadogo et al.,1993) as well as 28.03 g / l (Petit et Queval, 1973).
Low gamma globulin samples concentration could indicate the absence of microbial infections in animals at the time of collection.
The albumin / globulin ratio fluctuated between 0.93 and 1.07. This ratio is greater than that of Petit and Queval (1973) which varied between 0.54 and 0.56, as well as the 0.76 ± 18 (Sawadogo et al., 1993). Labouche (1964) reported values that ranged from 0.37 to 0.72, while those of Abouna (1990) varied between 0.60 and 0.75), which are lower than the present results. Futures research are needed for understanding the factors that might explain these differences.

Calcium

Calcium serum content varied between 2.25 and 2.67 mmol / l. Current results are higher than those of Zebu Choa (2.16 - 2.32 mmol / l) and lower than zebu Goudali (4.1 - 4.9 mmol /l) of Cameroon (Abouna, 1990). Friot and Calvet (1971) found an average content of 92.5 mg / l (88.6 - 95.1 mg / l), i.e. 2.31 (2.21 - 2.37 mmol / l) in Casamance during the dry season in Senegal, against 88.4 mg / l (84.3 - 98.9 mg / l or 2.20 mmol (2.10 - 2.47 mmol / l) in Ferlo in Senegal. Sawadogo et al. (1991) found a comparable value (2.35 mmol / l). These values are closer to the minimum value of this study. But, current study values are among reference values range indicated by the EISMV (nd) which vary between 2.25 and 3.00 mmol / l.

Phosphorus

Phosphorus serum levels varied between 1.38 mmol / l and 1.68 mmol / l. These values were lower than those reported by Abouna (1990) on Goudali (2.07 mmol / l) and Choa (2.08 mmol / l) zebus in Cameroon and those from Friot and Calvet (1971) in Ferlo, in the rainy season (66.0 mg / l or 2.13 mmol / l and 71.5 mg / l or 2.31 mmol / l, in Senegal. They are close to 49.4 mg / l or 1.59 mmol / l in the dry season in Ferlo, but lower than that obtained by these authors in 1973 (62.1 ± 0.9 mg / l or 2.0 ± 0.02 mmol / l). Habumuremyi (2007) found an average of 2.18 ± 0.93 in animals without pica and 1.93 ± 0.70 mmol in those with pica in Burkina Faso, which is higher than the present results. The present results are also lower than those of Sawadogo et al. (1991) in Senegal. These results confirm the low phosphorus level observed by Kouyaté et al. (1990) in the Malian Sahel. However, EISMV (nd) indicated that the reference values of phosphorus serum content are between 1.30 e t 2.10 mmol / l which fall within this study range.

Magnesium

Magnesium serum content varied between 1.07 and 1.77 mmol / l, which are close to the average (26.4 mg / l or 1.08 ± 0.01 mmol / l) obtained by Friot and Calvet (1973) in Senegal. However, these authors found in 1971, contents varying between 22.8 ± 0.9 and 25.7 ± 3.7 mg / l or 0.94 ± 0.07 and 1.06 ± 0.16 mmol / l, which are slightly lower than the present study findings. On the other hand, the findings of the present study are in the range of Habumuremyi (2007) values who reported an average of 0.91 ± 0.37 with values varying between 0.11 and 2.12 mmol / l in Burkina Faso. However, EISMV (sn) reported that the concentration reference varies between 12 mg / l and 35 mg / l, ie 0.49 mmol / l and 1.44 mmol / l, which is lower than the present results. Sawadogo et al. (1988) indicated the usual serum value of Mg is 18.2 ± 0.3 mg / l or 0.75 ± 0.01 mmol / l, which also is lower than those of the present study. Therefore, these values indicate that magnesium may not a constraint in the study area.

Zinc

Zinc Serum concentration fluctuated between 2.8 and 3.37 µmol / l. These values were lower than the 1.27 to 2.21 mg / l or 19.42 to 33.8 µmol / l observed by Friot and Calvet (1971). They were also lower than the 1.46 ± 0.03 mg / l or 22 , 33 ± 0.46 µmol / l reported by Friot and Calvet 1973) in Senegal, as well as those of Faye et al.(1986) on Ethiopian cattle (113.5 µg / 100 ml - 86 - 136 µg / 100 ml) i.e. 17.4 µmol / l (13.15 - 21.12 µmol / l). Sawadogo et al. (1988) indicated that the usual Zinc concentration in serum is 0.93 ± 0.02 mg / l or 14.22 ± 0.31 mmol / l in adult cattle, while Friot and Calvet (1973) indicated that European standards are between 0.60 and 1 mg / l, i.e. 9.18 and 15.29 µmol / l. Under these conditions, animals in the study area have zinc deficiency.

Copper

Copper serum varied between 2.18 and 2.69 µmol / l with an average of 2.68 ± 0.06 µmol / l. These values are lower than the 64.4 µg / 100 ml or 86 - 136 µg / 100 ml) or 10.16 µmol / l and 7.55 - 11.8 µmol / l found by Faye et al., 1986) on cattle in Ethiopia. There were also lower than the results of Friot and Calvet (1971) values in Senegalese regions: 0.51 ± 0.13 mg / l or 8.2 ± 2.05 µmol / l in Ferlo in the dry season, 0.62 ± 007 mg / l or 9.76 ± 1.10 µmol / l in the dry season and 0.5 ± 0.05 mg / l or 7.87 ± 0.79 µmol / l in Casamance. Sawadogo et al. (1988) indicated that the usual value for serum concentration of cupper is 0.72 ± 0.02 mg / l or 11.33 ± 0.47 mmol / l in adult cattle. In the other hand, Friot and Calvet (1973) indicated that the threshold for copper deficiency is 0.60 mg / l or 9.44 ± 0.16 µmol / l, while, the contents are between 0.75 and 1 mg / l or 11.80 and 15.74 µmol / l. Consequently, the animals in the study area suffer from copper deficiency, but more investigations should be conducted.

Autopsy findings

The main feature of cattle paralytic syndrome signs discovered in this study is the joint erosion of the forelimbs cartilage, especially the forelimbs. Previous studies (Kouyaté et al., 1988, 1995) have not reported this finding. In the absence of very expensive laboratory analysis, it is possible to diagnose the syndrome based on this characteristic on dead or slaughtered animals.

Trial 2

The patients healing rate treated with injectable solutions was 73% with 5 ml / 10 kg bodyweight dose, 86.2% with 1 ml / kg bodyweight dose and 100% with the lick stone. The 1 ml / kg bodyweight dose, administered every four days, is easier in practice, since it requires less veterinarian presence. These results indicate that the disease is indeed of mineral origin specially calcium and phosphorus, even if the levels of deficiencies have not yet been established. Treatment costs with veterinary products is relatively accessible to cattle breeders.

Results obtained in this study also make it possible to reject the hypothesis of botulism in the study area, contrary to what was reported in Ferlo in Senegal in the 60s and 70s (Calvet et al., (1965). Early clinically manifesting animals and in non-acute forms, the lick stone could save animals, but in the more serious forms, injections are essential. Treatment of mineral deficiencies is well documented in the literature. McDowell (1997) has therefore suggested several treatment methods according to deficiency types. In Senegal (Calvet et al., 1976;, Fall et al., 1999) used natural mineral salts such as phosphates or other sources of minerals to treat animals suffering from mineral deficiency. These methods could also be used in Mali, especially in the northern part of the country which is quite rich in phosphates. However, injectable products use is still valid in disease critical cases.

Conclusion

Cattle paralytic syndrome, observed in Mali since the 1985s, without identification of the real cause, is of mineral origin. Veterinary injectable products treatment used confirms that minerals in question are calcium, phosphorus and magnesium. In severe disease forms, doses of 0.5 ml / kg one day apart or 1 ml / kg bodyweight three days apart is recommended to the extension services pending other products availability. In non-acute forms, access to a quality lick containing calcium, phosphorus and magnesium helps to heal sick cattle. Cattle owners must be more informed and should be aware of awareness by the technical services to combat this disease. Otherwisse, other researches must to be conducted in order to clarify copper and zinc situation in the study area.

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

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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.

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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	

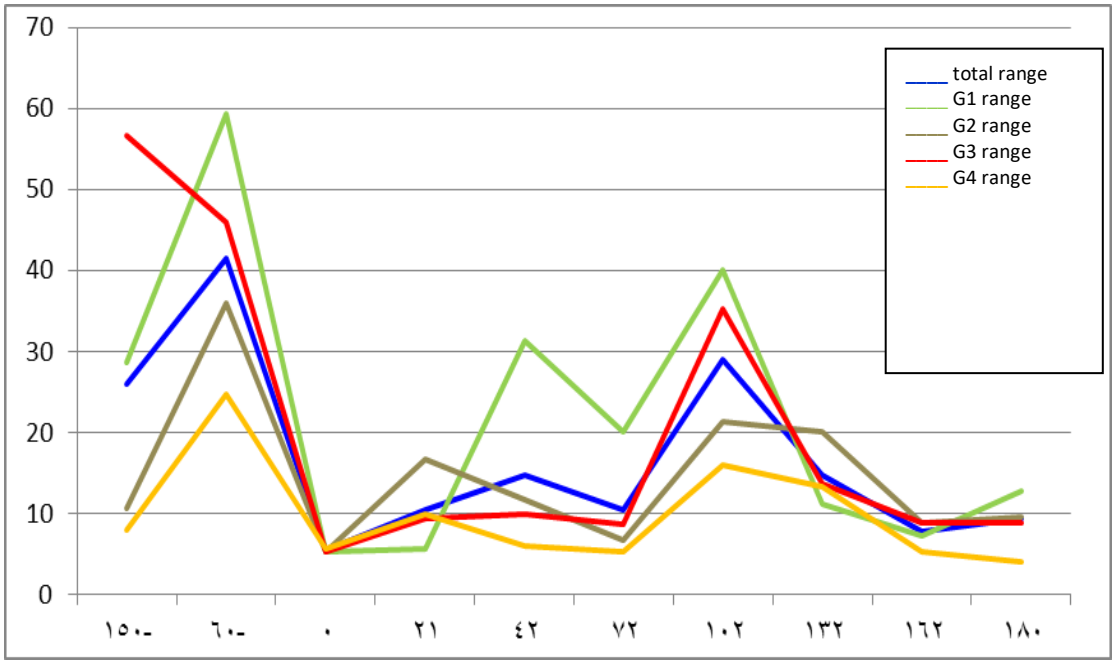

Figure. 1: The results of indirect hemagglutination test for all animals before and during the experiment.

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.

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