Comparison of ELISA with traditional methods used for evaluation of blackleg and gas gangrene vaccine

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Abstract: Potency test for bivalent clostridial vaccine against Blackleg and Gas gangrene mainly based on challenge test for estimation of bacterial immunogenicity of *Clostridium chauvoei*, and on serum neutralization test or hemolysin test for the *Clostridium septicum* toxigenicity. In this study, ELISA was done for evaluation of immunogenicity of Blackleg and Gas gangrene vaccine; three batches of vaccine were evaluated in guinea pigs, rabbits, and sheep. Challenge test; plate agglutination test, and indirect ELISA were done for *Clostridium chauvoei*, on the other hand, serum neutralization test; Hemolysin test, and Indirect ELISA were carried out for *Clostridium septicum*. The results obtained by ELISA pass parallel with challenge test, plate agglutination test, and Hemolysin test and there is no significant difference between them. Also there is great reproducibility in the results obtained in three batches. Also alpha toxoid of *Clostridium septicum* containing vaccine can be evaluated in guinea pigs, and there is great correlation between units obtained in sera of guinea pigs and that obtained in rabbits. So it concluded that the batch potency for *C. chauvoei* and *C. septicum* could be done by ELISA and replaced the traditional methods for evaluation due to precision, sensitivity, rapid, and save animals used.

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1. Introduction

C. chauvoei is an endospore forming Gram positive anaerobic bacterium which is the pathogenic agent of a disease called blackleg **(Hamaoka and Terakado, 1994)**. Blackleg is a disease causing serious toxemia associated with spore contaminated soil **(Sasaki *et al*., 2000).** It considered being fatal disease of cattle, sheep and other ruminants and wild animals and it is the most important clostridial disease producing economic losses in livestock industry **(Smith and Williams, 1984)**.

C. septicum is a Gram positive motile spore forming anaerobic bacterium that is implicated as the cause of traumatic and non-traumatic gas gangrene which is rapidly fatal disease **(Keogh *et al*., 1994).** Many aspects of *C. septicum* induced non-enteric disease in domestic animals **(Gyles, 1993).** Wound infection by *C. septicum* in animals is generally referred to as what is called malignant edema and usually followed direct contamination of traumatic wound **(Macleman, 1962).**

Immunity to both *C. chauvoei* and *C. septicum* is generally anti-bacterial and also anti-toxic. In malignant edema, the course of disease is rapid therefore the prevention of *C. septicum* induced infection is preferable than treatment. Commercial bacterins, toxoids and bacterin toxoids are available as vaccines usually consist of inactivated liquid cultures eliciting antibody responses to both bacterial surface antigens and toxic components **(Cygan, 1990; Hjerpe, 1990; Gyles, 1993).** This vaccine usually produces lifelong immunity **(Green *et al.,* 1987; Morgan *et al.,* 1988).**

In Blackleg disease unlike other clostridial infections, the predominant role in protection depends on cell wall, and flagellar antigen mainly **(Smith and Holdeman, 1968; Chandler and Gulasekharan, 1974).**

The potency test of the locally manufactured clostridial vaccines containing the *C. chauvoei* based on the challenge assay in guinea pigs as the vaccines must protect at least 85% of guinea pigs when challenged with either virulent strain or spore suspension of C. *chauvoei* **(Hamaoka *et al.,* 1990*;* British Pharmacopoeia, 2010).** On the other hand the potency test of the locally manufactured clostridial vaccines containing *C. septicum* antigens is mainly dependent on the measurement of the antitoxic immunity either by serum neutralization test in mice or by the haemolytic assay as **(British Pharmacopoeia, 2010*).***

Makhareta and Hammam, 2001 applied an enzyme linked immunosorbent assay (ELISA) for evaluation of immunogenicity of blackleg disease vaccine and found that good correlation between results of ELISA and challenge test. **(Osman *et al*., 1997)** studied the presence of antibodies in cattle sera previously vaccinated with blackleg vaccine by using plat agglutination test, indirect haemagglutination test, complement fixation test and enzyme linked immunosorbent assay, where they found that all these tests gave best results with no great difference

between them. While the application of ELISA in the measurement of immune antibodies to *C. septicum* will measure both the antibodies against alpha toxin of *C. septicum* and the antibodies against the bacterial surface antigens because the ELISA plates were coated by using the whole culture filtrate which containing both bacterins and toxins.

ELISA is suitable for measuring the immune response to different antigenic fractions instead of challenge or serum neutralization test because it has two advantages:- First, it will allow a significant reduction in the number of used guinea pigs or mice. Second, ELISA allow on open ended fully quantitative estimation of potency for vaccines **(Makhareta and Hammam, 2001).**

So the aim of this work is to use indirect ELISA to evaluate three vaccine batches of Blackleg and Gas gangrene and compare it with traditional methods used in evaluation of this vaccine.

2. .Material and Methods

1:- Vaccine preparation for C. chauvoei and C. septicum:-

Three batches of bivalent blackleg and gas gangrene vaccines (C. chauvoei and C. septicum) were prepared according to **Gadalla et al., 1974.** And subjected to sterility, safety before using them in immunization according to **British Pharmacopoeia, 2010)**

2:- Preparation of antigens:-

2-1:- Preparation and titration of C. chauvoei agglutinating antigen:-

It was prepared according to **(Claus and Macheak, 1972).**

2-2:- Preparation of C. chauvoei antigen for ELISA:-

It was prepared according to **(Matter et al., 2002).**

2-3:- Preparation and titration of C. chauvoei spore suspension

It was prepared according to **(Cooper et al., 1960).**

2-4:- Preparation of C. septicum antigen for ELISA:-

It was prepared according to **(Hong et al., 2002).**

3:- Antisera:-

3-1:- Positive sera:-

C. chauvoei and C. septicum positive sera were supplemented by Anaerobic Vaccine Research Department, VSVRI, Abbasia, Cairo, Egypt.

3-2:- Negative Sera:-

C. chauvoei and C. septicum negative sera were collected from non immunized animals (guinea pigs, rabbits, and sheep).

4:- Animals:-

4-1:- Guinea pigs:-

A total number of 60 Guinea pigs weighting 250-300 gm were used, they were divided into three groups, twenty G. pigs for each vaccine batch.

4-2:- Rabbits:-

A total number of 21 bosket rabbits weighing about 2-3 kg. were divided into three groups, seven rabbits for each vaccine batch.

4-3:- Sheep:-

Fifteen sheep of 9-12 month old were divided into three groups, five sheep for each vaccine batch.

4-4:-Mice:-

Swiss mice weighting between 15-20g were used in determination of test dose of alpha toxin, and in serum neutralization test of sera of immunized animals.

Vaccination schedule:

All the above mentioned animals were vaccinated S/C in two doses with 3 weeks intervals, where the 1st dose of vaccine was 3ml, and 2nd dose 2ml. then blood samples were taken after 2 weeks from 2nd dose, sera separated individually and part of them pooled for serum neutralization test.

5- Detection of antibody titers:-

5-1:- Detection of antibody titers against antigens of C. chauvoei

5-1-1:- Challenge test was carried for all vaccinated guinea pigs of the three groups according to (**British Pharmacopoeia 2010)**

5-1-2:- Serum samples of all vaccinated animals (guinea pigs, rabbits, and sheep) were exposed to:-

1- Plate agglutination test according to (**Claus and Macheak, 1972),** and the results was calculated according to equation:
(1/serum volume) ÷ (2* serum dilution) according to (**Troxel et al., 1997**)

2- Indirect ELISA test according to **(Wood, 1991; Matter et al., 2002)**

5-2:- Detection of antibody titer against antigens of C. septicum:-

All serum samples of all vaccinated animals (guinea pigs, rabbits, and sheep) were exposed to:-

1- Serum neutralization test according to **(British Pharmacopoeia, 2010)**
2- Hemolysin test according to (**Moussa, 1958).**
3- Indirect ELISA test according to **(Wood, 1991;Matter et al., 2002)**

5-3:-Determination of ELISA titers for *C. chauvoei* and *C. septicum*

It was done according to **(Grabowska et al., 2002**) where A relationship between ELISA absorbance of different dilutions of positive control serum (Y – axis) and corresponding units of these dilutions by mouse neutralization test or agglutination test (X – axis) made an equation ($Y= \alpha + \beta X$) where X = units of Positive control serum and α and β

(intercept and slope) fixed data, and it was calculated according to equation:

$$\beta = \frac{\sum xy - \frac{1}{(n)(\sum[x])(\sum[y])}}{\sum X^2 - \frac{1}{n(\sum[X])^2}}$$

$$\alpha = \frac{\sum y - b(\sum[x])}{n}$$

3. Results and Discussion

ELISA assay for measurement of antibody titer in serum samples are important for vaccine titration instead of serum neutralization test in mice. Many studies have described a comparison of antibody units from the results obtained from ELISA (**Reizenstein *et al*., 1995; Grabowska *et al*., 2002**).

The results of challenge test for *C. chauvoei* in vaccinated guinea pigs were illustrated in table (1) where the protection percent were 100% in G. pig challenge with 32 and 64 MLD of spore suspension. This results was passed with (**British Pharmacopoeia, 2010**) when accepted vaccine containing anaculture of *C. chauvoei* when passed challenged by 1 MLD of spore suspension of *C. chauvoei*.

Table (1): Surviving rate of vaccinated G. pigs with Bivalent Blackleg and Gas Gangrene vaccine against Challenge by spore suspension of *C. chauvoei*.

Spore suspension of *C. chauvoei*	No of G. pig	Batch (1)		Batch (2)		Batch (3)	
		Survive Animal	Survive %	Survive Animal	Survive %	Survive Animal	Survive %
32 MLD	5	5/5	100	5/5	100	5/5	100
64 MLD	5	5/5	100	5/5	100	5/5	100
128 MLD	5	4/5	80	4/5	80	4/5	80
256 MLD	5	4/5	80	3/5	60	4/5	80

Agglutination titer of *C. chauvoei* measured in sera of vaccinated g pigs in 3 batches of vaccines preparation as shown in table (2) the mean agglutination titer were 153.75, 143.75, and 143.75 in Batches (1,2,3) respectively.

There is no significant change between agglutination titer in three batches, and this result agree with (**Claus & Macheak, 1972**) which shown that there is correlation between agglutination titer and challenge test in g pigs and can be replaced the challenge test with plate agglutination test for saving lab animal.

Table (2): Mean agglutination Antibody titers against *C. chauvoei* in sera of vaccinated G. pigs, rabbits and sheep with Bivalent Blackleg and gas gangrene vaccine.

Vaccine Batches	G. pigs	Rabbits	Sheep
Batch (1)	153.75 ± 56.05	39.28 ± 12.37	80 ± 24.49
Batch (2)	143.75 ± 54.12	42.85 ± 11.29	80 ± 24.49
Batch (3)	143.75 ± 44.63	42.85 ± 11.29	80 ± 24.49

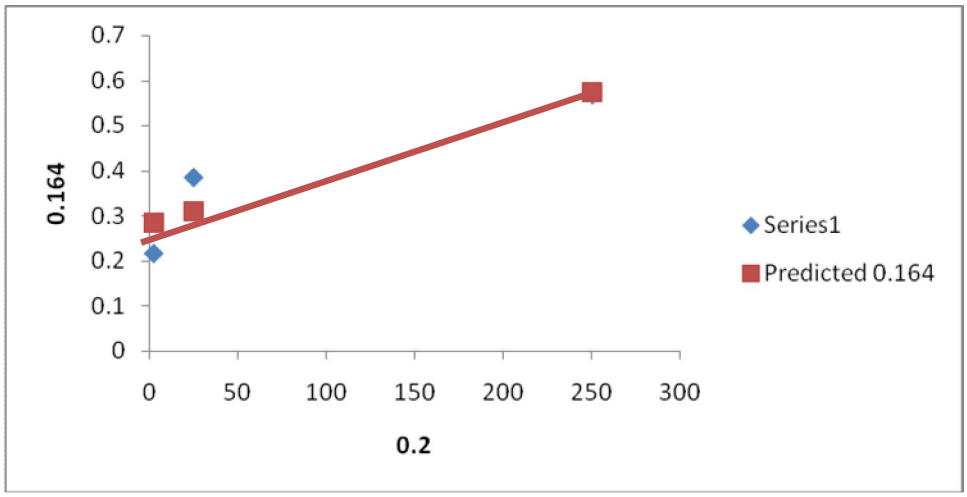
Assays for measurement of antibody responses in serum samples using immunoassays are important diagnostic and epidemiological tools for a variety of purposes (**Grabowska *et al*., 2002**). Many studies have described comparisons of different methods for calculating antibody units from the results obtained by ELISA (**Reizenstein *et al*., 1995**) and different mathematical models for construction standard or calibration curves (**Lagergard *et al*., 1988**). So in this study obtained standard curve was calculated by Linear Regression method according to **Freund, (2001)** which is useful and important not only because many relationships are actually of this form, but also because they often provide close approximations to relationships that would otherwise be difficult to described in mathematical terms. The term linear equation arises from the fact that the graph of y= a +bx is a straight line. That is all pairs of values of x and y that satisfy an equation of the form y= a +b x constitute points that fall on a straight line. The values of a and b are usually estimated from observed data, and once they have been determined can be calculated the unknown value of x when known the corresponding value of y. These results was shown in tables (3, 5) were a linear curve was done for relation between optical density obtained by ELISA and known samples previously measured antibody titer either by plate agglutination test, serum neutralization test, then when obtained the values (a, b) in the equation the unknown samples was

calculated in the equation and shown the results of antibody titers in serum samples of g pigs, rabbits, and sheep as shown in figures (1- 6).

The response of antibody titer against alpha toxin of *C. septicum* in sera of vaccinated rabbit were higher than that in sera of sheep as shown in table (4), this result come in accordance with (**Frerichs and Gray, 1975**) who found that rabbits responded as well as or better than sheep to the *C. septicum* component.

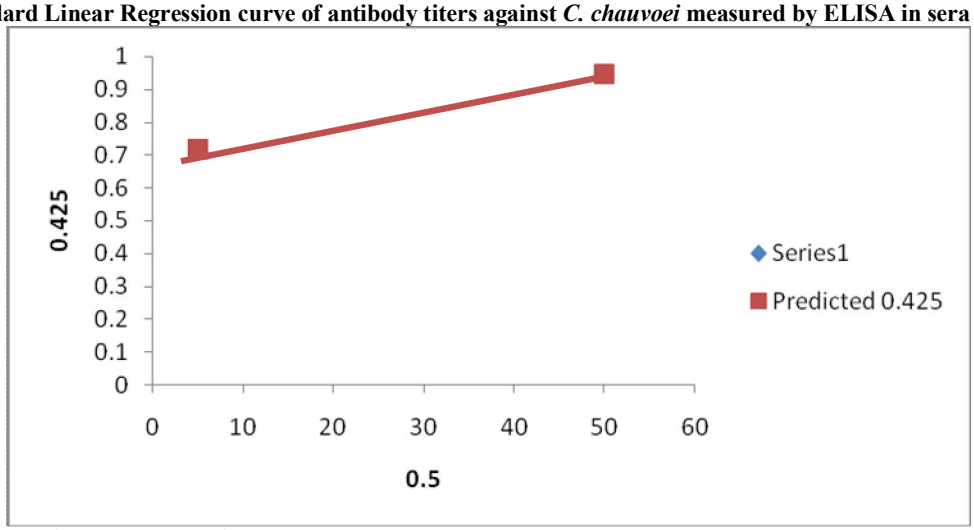
There is a great correlation between the results obtained by plate agglutination test, challenge test, Hemolysin test and results obtained by ELISA and also the reproducibility of the results obtained in different batches refer to be replaced the traditional methods for evaluation of Blackleg and Gas Gangrene vaccine with ELISA assay and calculated the results by linear regression method. And can be used only either guinea pig or rabbit for evaluation of vaccine. These results agree with (**Reizenstein *et al.*, 1995**) who compared five calculation modes for antibody ELISA procedures using pertussis serology as the model and found that the highest reproducibility combined with a high diagnostic sensitivity was achieved when the reference line model was used.

Fig. (1): Standard Linear Regression curve of antibody titers against *C. chauvoei* measured by ELISA in sera of G. pigs.



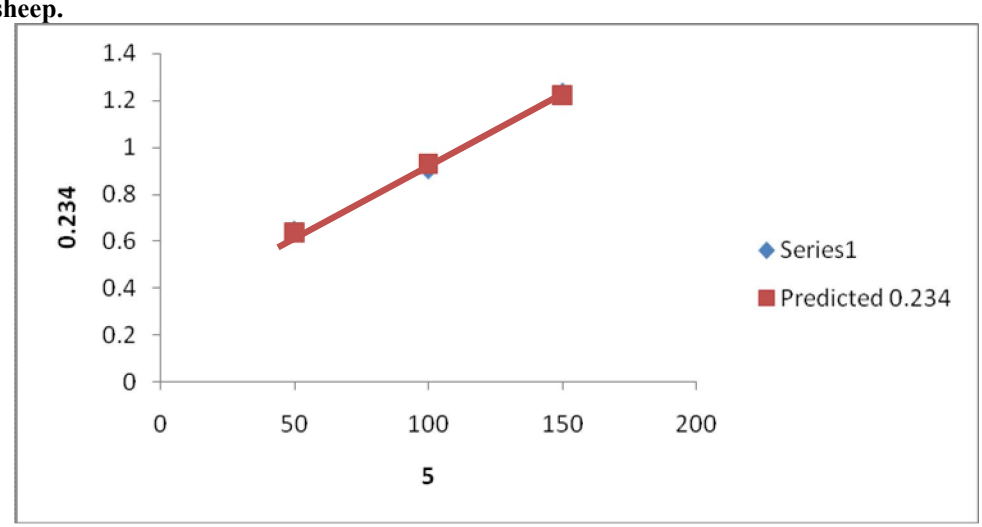
Y= (α) + (β) X [α=0.2391 β=0.00135]

Fig (2): Standard Linear Regression curve of antibody titers against *C. chauvoei* measured by ELISA in sera of rabbit.



Standard curve: α +β X [α(0.542) β(0.00833)]

Fig. (3): Standard Linear Regression curve of antibody titers against *C. chauvoei* measured by ELISA in sera of sheep.



Standard curve: α +β X [α(0.243) β(0.0067)]

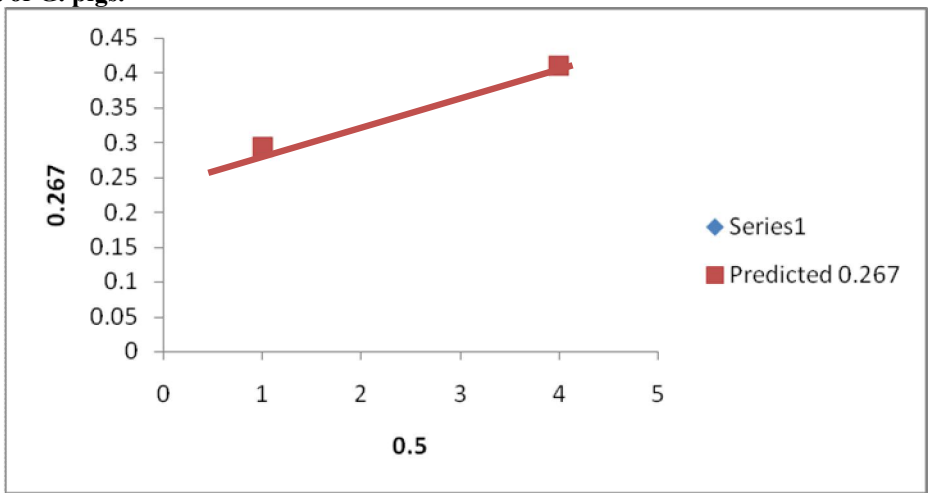
Table (3): Antibody titers against *C. chauvoei* in sera of vaccinated G. pigs, Rabbits and Sheep measured by ELISA.

Vaccine Batches	G. pigs	Rabbits	Sheep
Batch (1)	202.35 ± 39.11	42.66±23.01	77.54 ± 21.98
Batch (2)	198.95 ± 47.66	49.98±18.87	82.26 ± 24.96
Batch (3)	203.05 ± 36.05	48.4±17.54	87.33 ± 10.64

Table (4): Antibody titer against alpha toxin of *C. septicum* in sera of G. pigs, Rabbits and Sheep vaccinated with bivalent Blackleg and Gas Gangrene vaccine batches.

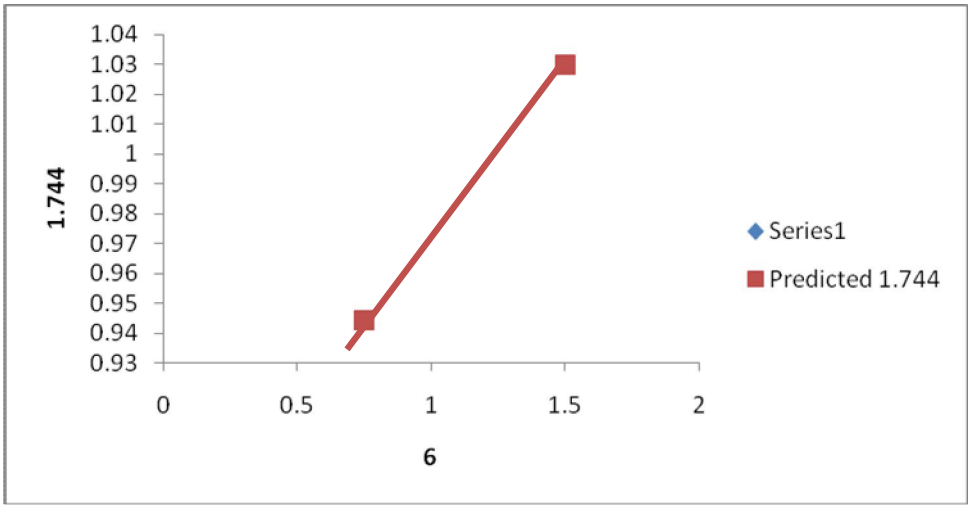
Vaccine Batches	G. pigs	Rabbit	Sheep
Batch (1)	4 IU	6 IU	4 IU
Batch (2)	4 IU	4 IU	4 IU
Batch (3)	4 IU	6 IU	4 IU

Fig. (4): Standard Linear Regression curve of antibody titers against alpha toxin *C. septicum* measured by ELISA in sera of G. pigs.



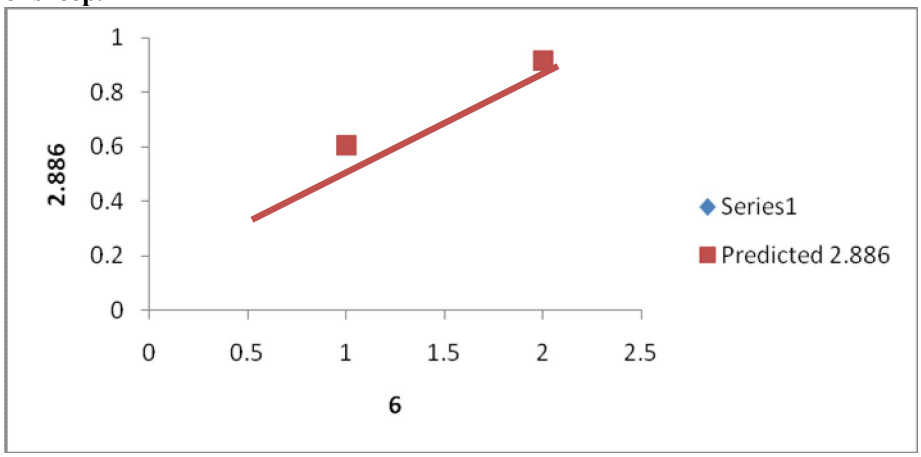
Standard curve: α +β X [α (0.2496) β (0.04020)].

Fig. (5): Standard Linear Regression curve of antibody titers against alpha toxin *C. septicum* measured by ELISA in sera of rabbit.



Standard curve: α +β X [α(0.81425) β(0.1545)].

Fig. (6): Standard Linear Regression curve of antibody titers against alpha toxin *C. septicum* measured by ELISA in sera of sheep.



Standard curve: α +β X [α(0.0672) β(0.4669)].

Table (5): Antibody titer against alpha toxin of *C. septicum* in sera of G. pigs, rabbit and sheep vaccinated with bivalent Blackleg and Gas Gangrene vaccine measured by ELISA.

Vaccine Batches	G. pigs	Rabbit	Sheep
Batch (1)	4.75 ±1.78	6.67 ±1.46	5.59 ±0.75
Batch (2)	4.23 ±0.91	6.23 ±0.36	5.26 ±0.93
Batch (3)	4.46 ±0.95	6.79 ±0.47	5.4 ±0.788

Table (6): Antibody titer against alpha toxin of *C. septicum* in sera of G. pigs, rabbit and sheep vaccinated with bivalent Blackleg and Gas Gangrene vaccine measured by Hemolysin test.

Vaccine Batches	G. pigs	Rabbit	Sheep
Batch (1)	2 HU	2 HU	4 HU
Batch (2)	2 HU	2 HU	4 HU
Batch (3)	2 HU	2 HU	4 HU

HU=Haemolysin Unit

From results obtained in this work, it could be concluded that the potency for *C. chauvoei* and *C. septicum* can be done by ELISA and replaced the traditional methods for evaluation due to its precision, sensitivity, rapid, and save animals used.

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