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THE USE OF PREPARED LATEX MICROBEADS FOR RAPID DETECTION OF BETA TOXIN OF *CLOSTRIDIUM PERFRINGENS* TYPE C IN CALVES

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ABSTRACT

The objective of the present work was to develop a rapid test {Latex} for detection and typing of beta toxin of toxigenic *Clostridium perfringens* (*C. perfringens*) type C in calves, which is the etiological agent of enterotoxaemia for several animal species. Anti- beta toxin "hyper immune serum (HIS)" was prepared in rabbits for coating microbeads. Agar gel precipitation test (AG PT) and indirect enzyme linked immuno-sorbent assay (i ELISA) were used for detection and titration of the prepared HIS, respectively. Fecal samples were collected from 100 diseased and 20 apparently healthy calves of different age and sex. Moreover, samples from 60 internal organs (intestine, liver, kidney, lung and heart) were collected from slaughtered and dead calves. The samples were examined by using of antitoxin-coated latex microbeads using (sLAT), where 99 out of 120 examined fecal samples (82.5%) and 31 out of 60 samples of the internal organs, (51.7%) were positive. The results demonstrated the suitability and reliability of LAT as a rapid field test for monitoring of the beta toxin of toxigenic *C. perfringens,* type C in sudden death outbreaks since it is sensitive, rapid and easily used technique.

Keywords:

Beta toxin, *C. perfringens*, Enterotoxaemia, Hyper immune serum, Latex agglutination test.

INTRODUCTION

C. perfringens is a Gram positive, spore-former and anaerobic bacterium that causes diseases in animals and human. It is commonly found in soil, sewage and water as well as in the intestines of both man and animals as normal inhabitant microorganisms **(Songer, 1996)**. The pathogenicity of this organism is attributed to their exotoxins **(Meer and Songer, 1997).**

These bacteria are normal inhabitant of the intestine, but are usually present in low numbers. They produce little toxins and under normal condition are removed by normal gut movements or are inactivated by circulating antibodies. Sudden changes in diet rich in carbohydrate as grazing lush, rapidly growing pastures or young cereal crops or heavy grain feeding (as in feedlots) enable the bacteria to multiply rapidly. Toxemia occurs when the movement of food in the intestine slows or the organisms multiply and produce toxin faster than can be removed or neutralized. Large amounts of toxins as well as large numbers of *C. perfringens* can usually be observed in the intestinal contents of diseased or dead animals **(Quinn *et al,* 1994 and Songer, 1996)**. *C. perfringens* has been classified into five toxigenic types (A, B, C, D, and E) based on the expression of four major toxins: alpha, beta, epsilon, and iota. Beta toxins, produced by *C. perfringens* (types B and C) induce fatal enteric disease in cattle, goats and sheep **(Bradley *et al,* 2013)**. Moreover, they are the etiological agent of enterotoxaemia (lamb dysentery and pulpy kidney disease) of several animal species **(Blood *et al.,* 1983)**. *C. perfringens* type C is the most prevalent isolates in calves. As *C. perfringens* is a natural host of human and animal intestines, identification of the bacterium is not sufficient. The diagnosis of enterotoxaemia is usually based on clinical signs and pathological findings, but identification of toxins in the intestinal contents is necessary to confirm the diagnosis **(Ozturk, 1996) and (Ozcan and Gurcay, 2000)**. ELISA and Latex agglutination tests have been used for laboratory diagnosis of enterotoxaemia, especially in cases of sudden death outbreaks in calves. These tests are simple, of limited cost, and provide quantitative and accurate results for toxin-typing and differential diagnosis of *C. perfringens* types A, B, C, and D enterotoxaemia **(El- Idrissi and Ward, 1992) and (Martin and Naylor, 1994)**. Substantial interest and effort have been expended in establishing new test for rapid detection and typing of *C. perfringens* type C in the veterinary routine diagnostic laboratory and in the farm animals those are the objective of the present work.

MATERIAL AND METHODS

1- Animals:

a- Calves: 132 calves of different ages and sex.

b- Rabbits: four healthy rabbits of New Zealand breed, each of 2 kg body weight, were used for preparation of HIS against beta toxin according to **(The GUIDE LINES FOR,2014:THE**

PRODUCTION OF ANTIBODIES IN LABORATORY ANIMALS, University of California, Berkeley Animal Care and Use Program)

c- Collection of normal rabbit serum as control -ve.

2- Samples:

a- Fecal samples:

One hundred samples were collected from calves with suspected enterotoxaemia and 20 samples were collected from apparently healthy calves (control -ve).

b- Internal organs:

Samples from internal organs included intestine, liver, kidney, lung and heart and were collected from eight slaughtered calves and four recently died ones.

3- Reference toxins and antitoxins:

Reference toxins and antitoxins they were kindly provided from Serum and Vaccine Research Institute, Abbassia (Anaerobic Unit), Cairo, Egypt and used for preparation of HIS against beta toxin and as control positive in serological tests.

4- Indirect enzyme linked immuno-sorbent assay (i ELISA):

I ELISA was used for detection and titration of beta toxin according to **(El-Idrissi and Ward, 1992).**

5- Latex agglutination test (LAT):

LAT was used for detection of beta toxin in the collected samples according to Martin and **Naylor (1994)**. A slide latex agglutination test (SLAT) was performed on a glass slide by using 25 µl of coated latex with 25 µl of 10-fold serial dilutions of samples in PBS (PH 7.2) 0.5% BSA. The mixture was gently rotated; the agglutination was recorded after 3 min. the presence of C. perfringens beta toxin was monitored by the LAT by using the supernatant of the samples.

6- Agar gel precipitation test (AGPT):

AGPT was used for detection of the prepared HIS and beta toxin in the collected samples according to **Parija (2014).**

7- Preparation of hyper immune serum (HIS) against beta toxin

Blood samples were collected from rabbits before the beginning of the first inoculation as control negative. *C. perfringens* type C beta toxin was inoculated subcutaneously for each rabbit. First inoculums (3ml) of Freund's complete adjuvant (FCA) mixed with 0.2mg of

toxin, at 4 - 6 sites. The booster doses at 2nd, 4th, 6th and 8th week's intervals were 3ml of Freund's incomplete adjuvant (FICA) mixed with 0.2mg of toxin at different 4 - 6 sites. After one week of the last injection, 3ml blood samples were collected from each inoculated rabbit with 1-week interval, via heart puncture route for separation of sera **(Subramanayam *et al.*, 2000)**. The collected serum tested using agar gel precipitation test **(**AGPT**)** for checking the production of HIS against beta toxin. The white line of precipitation between the central wells (contained 10 µl of beta toxin) and the peripheral wells (contained 10 µl of sera from inoculated rabbits) in AGPT plates indicating the high levels of HIS. The obtained HIS from inoculated rabbits was kept at - 20°C until used.

8- Purification and concentration of HIS using the ammonium sulfate procedure (Donovan and brown, 1995):

The collected rabbit blood was centrifuged for 3 min at 9,000 rpm at 4°C. The supernatant was decanted into a 25 ml beaker containing the serum, and then it was placed on ice tray over a magnetic stirrer. Four ml saturated ammonium sulfate were added slowly and left for 1 to 2 hr. at 4°C to ensure precipitation of the antibodies. The solution was centrifuged for 5 min at 9,000 rpm, at 4°C; the supernatant was decanted into a beaker, while the pellet was retained in the centrifuge tube. The precipitate was re-suspended in a 10 ml volume of PBS and clamped at one end of the tubing using a dialysis clamp. The dissolved precipitate was filled to approximately one-half of the capacity and the tubing was closed with a clamp. Dialysis tubing was placed in a beaker containing PBS buffer and dialyzed at least for 3 hours at 4°C. The dialysis buffer was changed four times during dialysis and tested for the access of ammonia by barium chloride 1% until obtaining a purified HIS. The purified HIS was titrated by iELISA.

9- Coating of latex microbeads with the HIS of beta toxin

a- Preparation of Latex Beads:

Latex beads suspension of 0.8-µm diameter (Sigma, St. Louis, MO) was spun down in an Eppendorf tube and washed twice in buffer solution, pH 8, to remove the detergent and sodium azide, contained as preservative in the suspension supplied by the manufacturer.

b- Sensitization of latex beads:

Latex particles were sensitized with beta HIS as described by **(Martin and Naylor, 1994)**. Briefly, the washed latex particles pellet was re-suspended in phosphate-buffered saline

(PBS) for 10% latex suspension. The obtained suspension was diluted 1:20 using PBS [pH 7.8] containing 1% bovine serum albumin. Equal volumes of a diluted latex particle suspension and the prepared HIS of beta toxin were mixed and agitated vigorously on a vortex. After thorough mixing, the mixture was put in a shaking water bath at 50°C for 10 min and then centrifuged at 8000 rpm for 5 min. The obtained pellet was re-suspended in the diluent of PBS [pH 7.8] containing 0.5 % bovine serum albumin and 0.1% sodium azide. Control latex particles were sensitized with normal rabbit sera in the same manner. All the sensitized latex particles were stored at 4 °C until used.

10- Application of prepared latex:

A slide -latex agglutination test was performed on a glass slide by using 25 µl of coated latex with 25 µl of 10-fold serial dilutions of samples. The mixture was gently rotated; the agglutination was recorded within 3 min. The positive agglutination results indicated the presence of *C. perfringens* beta toxin.

11- Removal of cross-reactivity in the prepared antisera:

Each of the prepared antitoxin against specific toxin was incubated with different types of *C. perfringens* toxins at 37 °C for 1 hour then centrifuged at 1000 rpm for 10 minutes **(De Roe, *et al.,* 1987)**. This step was done to eliminate cross reactivity between different types of *C.perfringens* antitoxins since corresponding toxins absorbed undesirable antitoxins.

Results and discussion:

C. perfringens is an anaerobic bacterium incriminated as a cause of sudden deaths in farm animals and its pathogenicity is associated with the production of 4 major exotoxins (A, B, C, D, and E) on the basis of the expression of these toxins **(Meer and Songer, 1997 and Popoff, 2011)**. Since C. *perfringens* is a natural inhabitant in animal intestines, so identification of the bacterium is not sufficient as diagnostic technique. The diagnosis of enterotoxaemia is usually based on clinical signs and pathological findings, but identification of toxins in the intestinal contents is very necessary to confirm the diagnosis, **(Ozturk, 1996 and Ozcan and Gurcay, 2000).** The most widely used methods for toxin detection are indirect ELISA (iELISA) **(Giulazian *et al.*, 2008 and Hamouda *et al,* 2009)** and Latex test **(Martin and Naylor, 1994). Subramanayam *et al.,* (2000) and Garcia *et al*, (2013)**, recommended slide agglutination and mouse neutralization tests as reliable methods for detection of *C. perfringens* enterotoxins. In the present study, the prepared Latex microbeads were used

for detection of beta toxin of *Clostridium perfringens* type C, one of the most virulent enterotoxins **(Nillo, 1993 and Bradley *et al,* 2013),** in the collected fecal samples and organs from normal and diseased calves and compared with the results of iELISA and AGPT. Data illustrated in (Table 1) revealed that beta toxin of *Clostridium perfringens* type C was detected by sLAT test in 87 from 100 samples collected from diseased animals (87.0%). These results are in agreement with that of **Nillo (1993), Ozcan (2000), Vaikosen, and Ikgatua (2005).** The later mentioned authors explained that, the high concentrations of the beta toxins are produced from the fast multiplication *Clostridium perfringens* type C bacteria because of the sudden change in feeding regime especially in increasing energy content of the rations in well growing calves. Moreover, the digestive upsets impair the digestive peristaltic that helps the accumulation of the beta toxins and its absorption in high concentrations in blood stream causing enterotoxaemia and sudden death of calves.

Table (1): Prevalence of Beta toxin in fecal samples using sLAT, iELISA and AGPT

Fecal Samples		Beta toxin					
		+ve sLAT		+ve iELISA		+ve AGPT	
Animals	No	No	%	No	%	No	%
Diseased	100	87	87	96	96	60	60
App. Healthy	20	12	60	16	80	8	40
Total	120	99	82.5	112	93.3	68	56.7

The high rate of positivity for beta toxins in apparently healthy animals (60%) is explained by the presence of the bacterium as a normal inhabitant in the intestinal tract, where it produces little but detectable amounts of enterotoxins. However, the intestinal movement without appearance of any symptoms **(Giulazian *et al.*, 2008 and Popoff, 2011)** flashes them**.** As shown in (Table 2) the highest rate of positivity for beta toxin was found in samples from the intestine (83.3%), followed by the liver and kidney samples (58.3%), lung (33.3%) and heart (25.0%). This indicates clearly that, not all toxins present in the intestine disseminate to the internal organs, particularly the lung and lung, which showed the least rate of positivity. These results are in agreement with that obtained by other authors **(Quinn *et al,* 1994 and Songer, 1996)**.

Table (2): Prevalence of Beta toxin in internal organs samples using sLAT, iELISA and AGPT

Internal organs		Beta toxin					
		+ve sLAT		+ve iELISA		+ve AGPT	
Organs	**No**	**No**	**%**	**No**	**%**	**No**	**%**
Intestine	**12**	**10**	**83.3**	**11**	**91.7**	**7**	**58.3**
Liver	**12**	**7**	**58.3**	**9**	**75**	**5**	**41.7**
Kidney	**12**	**7**	**58.3**	**9**	**75**	**4**	**33.3**
Lung	**12**	**4**	**33.3**	**5**	**41.7**	**4**	**33.3**
Heart	**12**	**3**	**25**	**3**	**25**	**2**	**16.7**
Total	**60**	**31**	**51.7**	**37**	**61.7**	**22**	**36.7**

It is clear from the data depicted in both tables, that iELISA was the most sensitive test in detecting beta toxin of *Clostridium perfringens* type C, not only in diseased but also in apparently health animals, both in fecal samples and samples collected from the internal organs. The high prevalence of beta toxin either in fecal samples or in the internal organs when examined by iELISA could be attributed to its high sensitivity as a result of using specific and purified anti-beta toxin, as proved by **(Naylor, *et al.*, 1987**). Moreover, the ELISA method can detect toxin concentrations as low as 0.1 µg /ml of toxin at optical density (OD) of 405 nm and is therefore diagnostically beneficial in a clinical diagnosis setting **(Vaikosen and Ikgatua, (2005) and (Waggett *et al.,* 2010)**. On the other hand, the iELISA technique needs professional persons, special instruments and different reagents. For that reasons, this study was established to prepare a latex test for rapid detection beta toxin of *Clostridium perfringens* type C in fecal and internal organs of diseased and dead animals. It yielded slightly lower rates of positivity (82.5%) than iELISA (93.3%). This may be due to the presence of some cross reactivity **(De Roe, *et al.,* 1987)**. However, LAT showed significantly higher results than that of AGPT (56.7%). This indicates that, the LAT is sensitive enough and reliable to be used in monitoring of the Clostridial causes in sudden deaths outbreaks since it is rapid, easily used and sensitive technique. **(Martin and Naylor, 1994)**. Moreover, the *C. perfringens* toxin can be detected instantaneously (within 4 minutes).

Latex test can be used for investigation of large number of animals in short time. On the other hand, this test requires minimum number and amounts of reagents that could be used in the form of portable diagnostic solution for use in the farm premises. The procedure will improve the accurate diagnosis of sudden diseases due to enterotoxaemia in the farm animals and will help to discover further epidemiological and aetiological aspects of these diseases.

CONCLUSION

From the obtained results, sLAT test was found to be an easy, rapid, specific, accurate, low cost, field test, which does not need sophisticated apparatuses or professional persons. On the other hand, the iELISA technique needs specific apparatuses and chemicals, is time consuming and needs professional persons and specialized laboratory. Moreover, iELISA cannot be used as a field test, but it is used as a confirmatory test. The AGPT not only needs a large quantity of toxin and antitoxin but it also takes longtime yields fluctuated results.

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