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A study on some virulence factors of *C. perfringens* types A, B and D isolated from different sources

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Abstract

A total of (26) strains of *C. perfringens* types A,B and D isolated from cases of enterotoxaemia and necrotic enteritis (cattle, chicken, sheep and goat 7, 7, 6 and 6) respectively were examined for the detection of their haemagglutinating, haemolysine and lecithinase activities. The results showed that all type A strains of different species were highly haemagglutinin producer with cattle of (1/64 - 1/1024) while type B strain of cattle origin was weak haemagglutinin producer in comparison to type B sheep and goat origin while gave high haemagglutinin producer. All type D strain were highly haemagglutinin producer for all species except goat which was weak producer for haemagglutinin. All sheep type A strain were highly haemolysine producer with titer of (1/16) while only (2) type A of cattle origin and (1) of chicken origin in highly haemolysine producer. Type B strains were moderately haemolysine producer of titer (1/4 -1/8) except chicken which gave low haemolysine titer (1/2), also one strain of goat origin gave the same titer. Type D strains of cattle origin gave high haemolysin production titer (1/16) while type D strains of goat origin gave low haemolysin production titer. All tested strains were lecithinase producer.

Key words: *C. perfringens, enterotoxaemia, necrotic enteritis, haemolysin activity.*

Introduction

Clostridium perfringens is a Gram positive, spore-forming, anaerobic rod, Strains of *C. perfringens* are classified into five toxinotypes (A-E) based on the presence of four major toxin genes *(alpha, beta, iota and epsilon).* Mainly strains of type A are recovered from enterotoxemia cases but also from the intestine of healthy cattle and other warm-blooded animals as well as from the environment. *C. perfringens* type A has the ability to produce numerous extracellular toxins and enzymes, of which alpha toxin is the most toxic. Recently alpha toxin, a phospholipase C and perfringolysin O, a pore forming cytolysin, have been proposed as essential factors for induction of enterotoxemia. In addition to these toxins, other potential virulence factors might have a role in the pathology of enterotoxemia. Possible virulence traits can be proteolytic factors that suggested for necrotic enteritis in broilers . In a calf intestinal loop model, it has been shown that *C. perfringens* strains isolated from healthy and enterotoxemic cattle as well as from other host species are all capable of inducing necrohemorrhagic intestinal lesions . **(Evy Goossens *et al*., 2014)**.

Clostridium perfringens alpha-toxin is the major virulence factor in the pathogenesis of gas gangrene. *Clostridium perfringens*, are widely distributed in nature. It produce one PLC alpha -toxin that is highly cytotoxic and myotoxic and can lead to hemolysis and the release of superoxide radicals and inflammatory cytokines. The toxin has been associated with enteritis in domestic animals. **(Masataka Oda *et al*., 2012).**

Clostridium. perfringens haemagglutinin was partially diffusible and partially cell-bounded. The concentration of the haemagglutinin was

decreased from the supernatant after serial washing with saline, on other hand, much haemagglutinin was released from the cells by its disintegration ultrasonically and this proved that *C. perfringens* haemagglutinin contained intracellulary. All strains of *C. perfringens* types B and D produced haemagglutinin. Inhibition of *C. perfringens* haemagglutinin of types A, B and D by their corresponding antisera showed that the haemagglutinin of any type could neutralized and inhibited by the antisera of other types to variable dilutions. On other hand, many other heterologous clostridial antitoxic sera could not inhibit the haemagglutinin of all examined types. The haemolysin of *C. perfringens* serovars B and D were completely inhibited by their homologous and other heterologous clostridial antitoxic sera. **(El – Bardisy, 1988)**.

Some haemagglutinin of *Clostridium. perfringens* were more active against sheep than human erythrocyte. **(Mehta and Narayan 1991).**

Hemolysin produced by the *C. perfringens* isolates was detected by inoculating the bacterial colonies on sheep blood agar, as previously described **(McGinley 1998).** Inoculated plates were incubated at 37°C anaerobically for 24 hours. Presence of hemolytic zones (double or single) around colonies indicated production of hemolysin. Four components of theta toxin could be detectedeach hemolytic component was activated by cystine Hcl & inactivated by cholesterol & by heating at 60°C for 10 minutes **(Smyth 1975).** Also haemolysin can be detected in the broth culture as mentioned by **(Oakley and Warrack, 1953).**

The production of phospholipase C by the *C. perfringens* isolates was verified according to the literature **(Baldassi, *et al.*, 2002)** . Suspected colonies were inoculated into Wills and Hobb's medium without lecithinase-specific antisera, and added to neomycin sulfate at the concentration of 250 mg per mL. Plates were incubated anaerobically at 37°C for 24 hours and then examined for the presence of a pink zone of opacity around the lecithinase-positive and lactose-positive colonies **(Rahman M. *et al.*, 2012).**

The alpha -toxin is nevertheless a hazardous enzyme possessing phospholipase C as well as sphingomyelinase activity **(Titball and Basak, 2004).**

All the phospholipase C positive isolates revealed the presence of *cpa* gene encoding alpha toxin. **(Rahman *et al.*, 2012).**

The aim of the present work is the studying of haemagglutinine and haemolysine activities as virulence factors of *C. perfringens* strains isolated from different sources as well as determination of the lecithinase activity these strains.

Materials and Methods

1- Bacterial strains:

A total of 26 *C. perfringens* strains were obtained from the anaerobic unit at Animal Health Research Institute, the species, types and number of the strain are illustrated in **table (1)**. All strains were examined for detection of their haemagglutinin and haemolysin activities as well as lecithinase activity.

Table (1).

Source	No. of tested strain	Types		
		A	B	D
Cattle	7	5	1	1
Chicken	7	4	1	2
Sheep	6	3	1	2
Goat	6	3	2	1
Total	26	15	5	6

2- Haemagglutination test according to Wickham, 1956 and Collee, 1961:

A- Physiological saline (0.3 ml) was put in all the wells of the haemagglutination plate.

B- Whole culture or its supernatant (0.3 ml) was put in the first well and mixed well.

C- Serial dilutions from the first well was applied by transmitting 0.3 ml from it to the second ------ etc to reach the following final dilutions (1/2, 1/4, 1/8, 1/16,1/32, 1/64, 1/128, 1/256, 1/512 and 1/1024).

D- Thrice washed 1% sheep R.B.Cs. suspension in saline (0.2 ml) was added to each well and the constituents were mixed by handling rotation of the plate in a circular manner.

Two negative controls of normal saline (control 1) or sterile media (control 2) were tested in the same manner

E – Reading of the test:

The test was read after both controls had been settled.

Interpretation of the results:

Negative = the red cells formed a dense central button.

Positive = the red cells falled in a reticulum covered the base of the plate .

Decreasing degrees of haemagglutination were recorded as ++++, +++, ++ and +. The last well showed complete haemagglutination (+++) was considered to contain one haemagglutination unit for the amount of red cells used in the test.

3- Haemolysin test (Oakley and Warrack, 1953):

A- The supernatant culture to be tested for either *C. perfringens* type A, B and D was serially diluted in saline (1/2 – 1/1024).

B- Saline (0.5 ml) was added to each tube .

C- Thrice washed 2% sheep RBCs suspension in saline (0.5 ml) was added to each tube dilution and the mixtures were then shaked.

D- The mixtures were then incubated in water bath at 37°C for 1hr. and finally at room temperature overnight.

Negative control by using 0.5 ml of sterile media instead of the supernatant was tested in the same manner.

Interpretation of the results:

The results were interpreted as the last tube showed complete haemolysis of RBCs. and considered to contain one haemolytic unit which called the minimum haemolytic dose **(Oakley and Warrack, 1953).**

3- Detection of lecithinase activity (Nagler's Reaction)

The production of phospholipase C by the *C. perfringens* isolates was verified according to **(Willis AT 1977).** Suspected colonies were inoculated into Wills and Hobb's medium without lecithinase-specific antisera, and added to neomycin sulfate at the concentration of 250 mg per ml. Plates were incubated anaerobically at 37°C for 24 hours and then examined for the presence of a pink zone of opacity around the lecithinase-positive and lactose-positive colonies.

Results and Discussion

Clostridium perfrngens is widely distributed all over the world and caused severe losses for well nourished healthy animals, therefore, it could hinder animal production in Egypt, *C. perfringens* caused great losses in animals than was usually believed and wide spread epizootics were a feature of the disease**.** Lettle or no recent literatures have been dealt with haemagglutinin and haemolysin activities of *C. perfringens*. Rrgarding the haemagglutining activity type A strains of different species, the results illusterated in **table (2) & fig. (1)** revealed that all type A cattle strain are highly haemagglutining with average titre(1/64 - 1/1024) as well as sheep and goat strain while only one of chicken strains showed high haemagglutining production.

Table (3) & fig. (2) showed that type B strains of sheep and goat are highly haemagglutining producer while cattle type B strains showed lower haemagglutining production with average titre of (1/64 – 1/1024) and 1/4 – 1/16 respect nearly, on the other hand chicken type B strains was moderately haemagglutining producer with average titre (1/32 – 1/64). Variation in the haemagglutining activity type D

strains was noticed as shown in **table (4) & fig. (3)** where type D cattle strains was highly haemagglutining producer, while one of chicken type D strains was highly haemagglutining producer with average titre (1/64 – 1/1024) and one moderately haemagglutining producer, type D goat strains was weak producer for haemagglutination. These results are more or less agreed with that reported by **(Wickham, 1956)** who found that of the 14 old stock *C. perfringens* strains, some produced higher titers, other lower titers ranged from 1/2 – 1/256 and some were negative. **(Collee, 1961)** who found that several cultures of *C. perfringens* type A produced haemagglutinin. Also **(El – Bardisy, 1988)** reported that type B strains differed from types A & D where 100% of type B strains were haemagglutinin producer 76.5% of type D strains and 57.1% of type A strains were haemagglutinin producer. Toxigenic types of *C. perfringens* varied in their ability to produced haemagglutinin and also in end titer levels. Titer of haemagglutinin in culture supernatant was consistently lower than in cell extract **(Mehta and Narayan 1991).**

Clostridium perfrngens cattle type A strains were varied in their haemolysin activity (2) gave titre of 1/16 & (2) gave titre of (1/4 – 1/8) and one was weakly haemolysin producer 1/2. On other hand, all type A sheep strains were highly haemolysin producer (1/16) while that 2 of goat origin gave moderate haemolysin titre (1/4 – 1/8) and one strain was weakly haemolysin producer (1/2) chicken type A strains gave moderately titre of haemolysin (2) strains of cattle (1/4 – 1/8) and one strain for each of high haemolysin producer & weak haemolysin producer **table (5) & fig. (4).**

Regarding the haemolysin activity of type B strain recovered from cattle, sheep & goat gave moderately titre of haemolysin (1/4 – 1/8) and one strain for each of chicken and goat was weakly haemolysin producer **table (6) & fig. (5).** One strain of *C. perfrngens* type D strain of cattle origin was highly haemolysin producer with a titre of (1/16) while that of goat was weakly haemolysin producer with a titre of (1/2), in addition two strains one for each of chicken & sheep were of moderate haemolysin activity with a titre of (1/4 – 1/8) also two strains one for each of chicken & sheep were of weak haemolysin producer **table (7) & fig. (6). (Brooks *et al*., 1957)** found that the theta haemolysin was produced by types B & D in equal amounts by 50% of strains of both types. **(Bernhemier & Schwartz 1965) and Beth & Buttery (1978)** who recorded that the theta haemolysin was produced in greatest amount by types B & C than A, D & B types. Meanwhile **(El-Bardisy 1988)** observed that *C. perfrngens* type B & D produced haemolysin with variable end titer. The haemolysin production was greater in type B strains than type D ones where the titers ranged from 1/32 – 1/256 in type B strains & from 1/8 -1/64 in Type D ones. Also **(Rahman *et al*., 2012)** found that hemolysin production could be detected in 14 (29.16%) out of 102 examined samples, including 13 of non-avian origin. From these, ten were from clinically affected mammals, as was the bird isolate. Also a total of 28 (58.33%) isolates were positive for phospholipase C production and all were recovered from clinically affected birds and other animals. The high presence of hemolysin in the isolates was also reported in all the strains of *C. perfringens* type C isolated from cases of enterotoxemia in domestic animals **(Niilo, 1987).** All isolates of *C. perfringens* positive for phospholipase C production presented the a toxin gene, which is indicative of *C. perfringens* type A involvement in the disease of clinically affected animals **(Kumar *et al*., (2011).**

On the other hand, all examined *C. perfringens* strains of different source were positive for lecithinase activity. The lecithinase or alpha toxin of *Clostridium perfringens* is generally considered to be the most significant factor in the pathogenesis of toxemia caused by type A strains of this organism **(Paul 1961).**

Table (2). Haemagglutinin average type A strains isolated from different species:

Species	Haemagglutinin producers		
	High producer	Moderate producer	Weak producer
	1/64 - 1/1024	1/32 - 1/64	1/4 - 1/16
Cattle	5	-	-
Chicken	1	2	1
Sheep	3	-	-
Goat	3	-	-

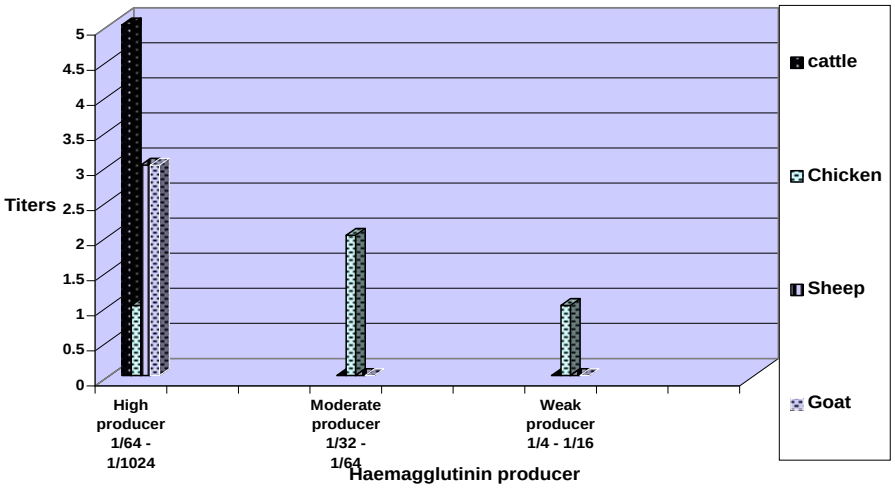

Fig (1) :Haemagglutinin average type A strains isolated from different species :

Table (3). Haemagglutinin average type B strains isolated from different species:

Species	Haemagglutinin producers		
	High producer	Moderate producer	Weak producer
	1/64 - 1/1024	1/32 - 1/64	1/4 - 1/16
Cattle	-	-	1
Chicken	-	1	-
Sheep	1	-	-
Goat	2	-	-

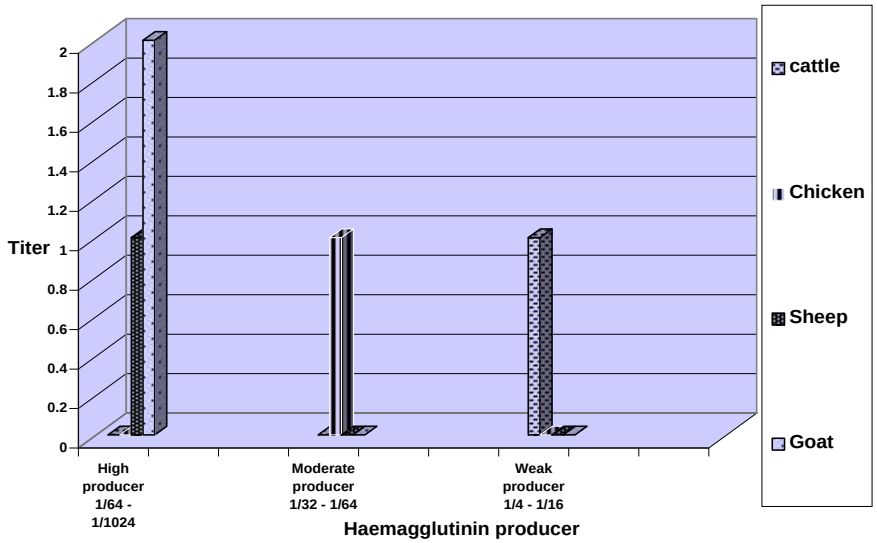

Fig (2) :Haemagglutinin average type B strains isolated from different species

Table (4). Haemagglutinin average type D strains isolated from different species:

Species	Haemagglutinin producers		
	High producer	Moderate producer	Weak producer
	1/64 - 1/1024	1/32 - 1/64	1/4 - 1/16
Cattle	1	-	-
Chicken	1	1	-
Sheep	1	-	1
Goat	-	-	1

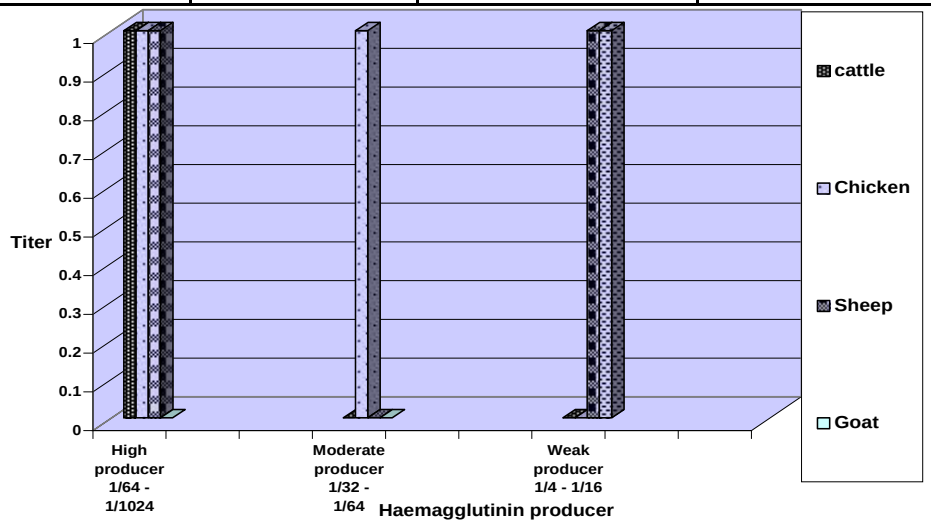

Fig (3) : Haemagglutinin average type D strains isolated from different species

Table (5). Variation in the haemolysin activity among type A strain:

Species	Haemolysin producers		
	High producer	Moderate producer	Weak producer
	1/16	1/4 - 1/8	1/2
Cattle	2	2	1
Chicken	1	2	1
Sheep	3	-	-
Goat	-	2	1

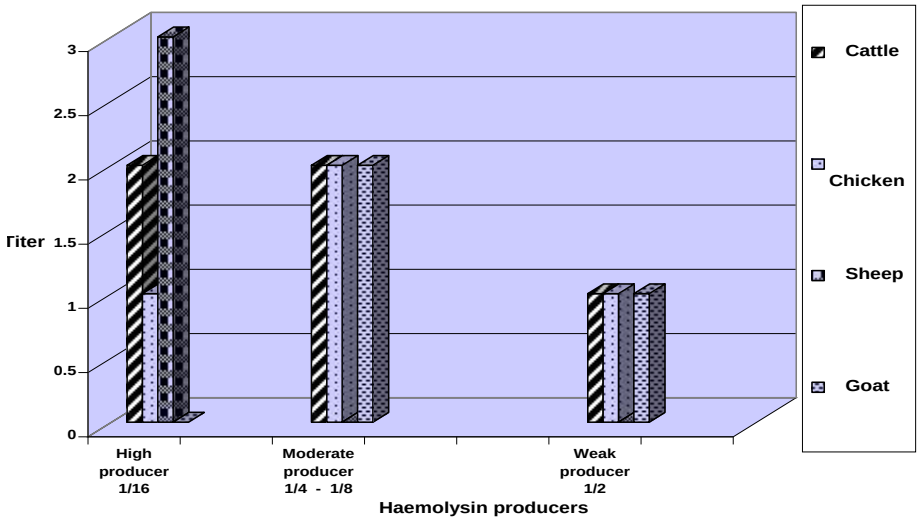

Fig (4) : Variation in the haemolysin activity among type A strain

Table (6). Variation in the haemolysin activity among type B strain:

Species	Haemolysin producers		
	High producer	Moderate producer	Weak producer
	1/16	1/4 - 1/8	1/2
Cattle	-	1	-
Chicken	-	-	1
Sheep	-	1	-
Goat	-	1	1

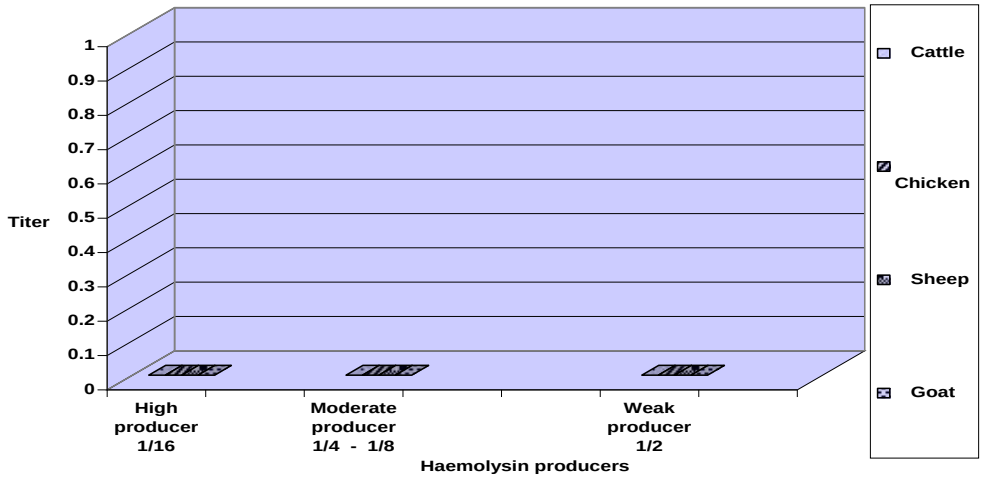

Fig (5) : Variation in the haemolysin activity among type B strain

Table (7). Variation in the haemolysin activity among type D strain:

Species	Haemolysin producers		
	High producer	Moderate producer	Weak producer
	1/16	1/4 - 1/8	1/2
Cattle	1	-	-
Chicken	-	1	1
Sheep	-	1	1
Goat	-	-	1

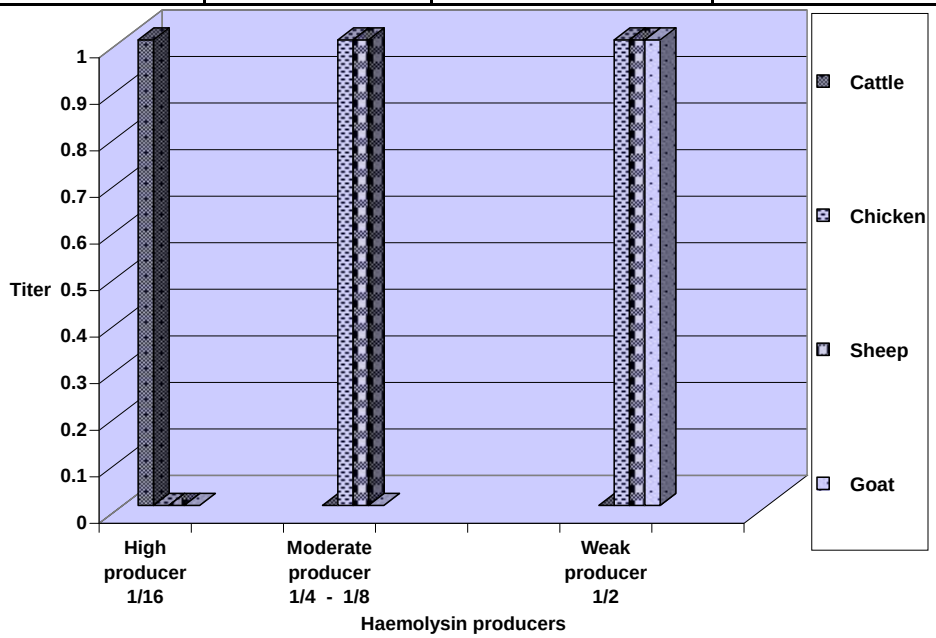

Fig (6) : Variation in the haemolysin activity among type D strain

Conclusion

Clostridium perfringens types A,B and D varied in their haemagglutinating activity, also there is variations between the same type strains isolated from different species.

This study proved that *C. perfringens* type A strains were highly haemagglutinating producer than type B strains. Type D haemagglutinating strains of cattle, sheep and chicken were highly than that of goat origen.

Type A strains of cattle, sheep, chicken and goat were highly haemolysin producer than type B ones while type D strains of cattle origin gave highly haemolysin production titers than type D of other species.

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دراسة عن بعض عوامل الضراوة لميكروب الكلوستريديم بيرفرينجينز نوع(أ)، (ب) و(د) المعزول من مصادر مختلفة

داليا اسكندر

وحدة اللاهوائيات . قسم البكتريولوجى .معهد بحوث صحة الحيوان بالدقى

الملخص العربى

اجريت هذه الدراسة على عدد (٢٦) عترة بكتيرية لميكروب الكلوستريديم بيرفرينجينز والمعزولة من الابقار – الدجاج – الاغنام – الماعز بواقع (٧- ٧ - ٦ - ٦) على التوالى لدراسة انتاج هذه العترات للمادة الملزنة للدم والمادة المحللة للدم والتأثير على وسط الاجار بالبيض. اظهرت النتائج ان جميع عترات النوع (أ) انتجت المادة الملزنة للدم بمستوى عالى (١/٦٤ – ١/١٠٢٤) بينما عترات النوع (ب) والمعزولة من الابقار اعطت مستوى منخفض للمادة الملزنة للدم (١/٤ - ١ /١٦) بالمقارنة بعترات لنفس النوع والمعزولة من الاغنام والماعز وقد اعطو مستوى عالى وكانت جميع عترات النوع (د) والمعزول من جميع المصادر وقد اعطت مستوى عالى من المادة الملزنة للدم ماعدا العترات المعزولة من الماعز والتى اعطت مستوى منخفض .

كانت جميع عترات النوع (أ) المعزولة من الاغنام ذات مستوى عالى من المادة المحللة للدم بينما اعطت عدد (٢) عترة فقط معزولة من الابقار بمستوى عالى وكذلك المعزولة من الدجاج جميع عترات النوع (ب) اعطت مستوى متوسط من المادة المحللة للدم (١/٤ - ١/٨) ماعدا المعزولة من الدجاج والتى اعطت مستوى منخفض .

اظهرت عترات النوع (د) معزولة من الابقار بمستوى عالى من المادة المحللة للدم بينما اظهرت عترات نفس النوع المعزول من الماعز مستوى منخفض للمادة المحللة للدم . من الناحية الاخرى كانت بجميع العترات التى تم دراستها ايجابية لاختبار الليسيسينيز بتأثيره على وسط الاجار بالبيض .