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APPLICATION OF MODERN AND RAPID METHODS FOR DIAGNOSIS OF BLACKLEG AND GAS GANGRENE DISEASES IN BUFFALO CALVES AND SHEEP

ELHAM I. ATWA* and MERVAT A.EL-RAHMAN ALI**

*Bacteriology Department, Animal Health Research Institute, Shebin El - Kom-Menoufiea and

**Animal Health Research Institute Dokki, Egypt.

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SUMMARY

A total of (160) samples of infected muscles and exudates were collected from (80) dead buffalo calves and (80) dead sheep, which showed clinical symptoms of blackleg and gas gangrene diseases. Samples were collected from different farms in Egypt. The bacteriological examination for clostridial gas gangrene organisms (*C. chauvoei, C. septicum* and *C. perfringens* type "A") yield 34 (21.3%) clostridial organisms. *C. chauvoei, C. septicum C.* and *perfringens* type "A" were obtained from buffalo calves with incidence of 8.8%, 6.2% and 11.3%, respectively .While the isolation rate from sheep was 3.8% , 5% and 7.5%, respectively .

ELISA (enzyme linked immunosorbent assay), was in agreement with mouse protection test, and used for diagnosis of (*C. chauvoei* , *C. septicum* and *C. perfringens* type "A") from culture.

Polymerase chain reaction assay (PCR) was a useful tool for direct and rapid detection of *C. chauvoei* , *C. septicum* and *C. perfringens* type "A" from clinical specimens (infected muscles with blackleg and gas gangrene). The 522 bp. amplification of *C. chauvoei* , 594 bp. amplification of *C. septicum*, while 1167bp. amplification of *C. perfringens* type "A".

The present study has shown that it is possible to amplify DNA specific to *C. chauvoei* and *C. septicum* in clinical materials as infected muscles and exudates in the same sample by PCR , and by one primer. But *C. perfringens* need another primer for rapid diagnosis in clinical materials by PCR .

INTRODUCTION

Clostridia were widely distributed in nature, soil , dust, air, water, sewege and manure (George and Finegold, 1985). Consequently almost any wound is liable to be contaminated with such organisms especially after shearing , castration, tail doking, surgical operation or parturition (Smith and Holdeman, 1968 and Rue Jensen and Swift, 1982).

More than 20 strains of clostridia are involved in gas gangrene (Willis, 1969). Clostridial myonecrosis (gas gangrene)in the form of blackleg and malignant oedema is a fatal infection of the skeletal muscle and subcutenous tissue, and a rapidly progressive life threatening condition, resulting in the liquefication necrosis of muscle, gas formation associated with clinical signs of toxaemia and a high level of mortality (Sasaki et al., 2000b) .

C. chauvoei is the causative agent of blackleg disease, a strictly anaerobic, endo- spore, gram- positive bacterium considered to be a soil organism which mainly affects bovines and ovines and is responsible for significant economic losses. In sheep, the disease more frequently presents as a wound infection resembling malignant oedema (*C.septicum* infection) or gas gangrene (*C. perfringens* infection), as conculded by (Smith and Holdeman, 1968) .

The differential diagnosis for gas gangrene organisms should consider the clinical data and necropsy lesions, microscopic, cultural and biochemical features, isolation of the agent, neutralization of lethal toxins in mice and guinea pigs using specific antisera and immunological methods such as immunofluorescence staining, (Homaoka and Terakado, 1994 , Kuhnert et al., 1997 and Pinto et al., 2005). However, these diagnostic methods are time consuming and laborious. Thus there is a great need for simple methods for detection and identification of pathogenic clostridia in gas gangrene (Sasaki et al., 2000a).

Several laboratory methods have much improved in recent years the development of immunological especially (ELISA) and genetic espeically (PCR) assays used to identify these organisms.

Enzyme linked immunosorbent assay (ELISA) is sensitive and qualitative, it allowes the differential diagnosis of clostridial species isolated from blackleg disease and gas gangrene (Poxton and Byrne, 1984). Also ELISA has developed as an alternative to neuteralization test in mice for detecting *C. perfringens* α toxin (Naylor et al., 1997)

Nucleic acid amplification of a specific target region of the bacterial genome by the polymerase chain reaction (PCR) is becoming widely used for detection and diagnostic purposes (Whelen and

Persing, 1996 and Vaneechoutte and Van Eldere, 1997).

PCR has been used for detection of alpha toxin of *C. septicum* (Takeuchi et al., 1997), and the 16S rRNA genes of *C . Chauvoei* and *C. septicum* (Kuhnert et al., 1997), or that the pathogens be differentiated by restriction enzyme digestion of the reducing PCR products. Polymerase chain reaction amplication of the 16S -23S rDNA spacer region genes has been suggested as the basis of a universal bacterial identification and typing system .

It may be easy to identify pathogenic clostridia by PCR using the common primers designed from highly conserved region sequences of the 16S rRNA and the 23S rRNA genes (Uzal et al., 2003). *C. chauvoei* was isolated in pure culture and detected by a fluorescent antibody technique (FAT)and a polymerase chain reaction (PCR) techinque (Sasaki et al., 2000a and Assis et al., 2002). The alpha toxin gene PCR is suggested as a diagnostic method for confirmation of *C. perfringens* species , providing a good alternative to the time consuming and specific mouse neuteralization test normally used in laboratory routine (Baums et al., 2004 and Piatti et al., 2004) .

This study was performed to investigate isolates and aid in rapid differential diagnosis between anaerobic blackleg and gas gangrene organisms by using different tests such as ELISA and PCR tests in order to obtain a better method to substitute the in vivo test .

MATERIALS AND METHODS

Samples:

A total of (160) infected muscles and exudates samples were collected from dead animals (80 sheep and 80 buffalo calves) which showed clinical symptoms of blackleg and gas gangrene. Post mortem findings observed including , extremely enlarged of the thigh , and distended with gas with marked crepitation felt by pulpation to the hock . The infected muscles were very dark reddish brown to black in colour and spongy due to gas bubbles within it . The infected muscles had rancid butter odour of blackleg muscles . Samples were collected from different farms in Egypt .

Bacteriological examination:

Gram- stained impression smears on affected muscles can yield some useful information about the morphology of the blackleg and gas gangarene organisms .

All samples were subjected to bacteriological examination for anaerobes. Cultural were made from infected muscle lesions and from exudate by sterilizing the exposed surface of affected muscle and cutting down a small piece from the deeper parts, and inoculated into Robertson's cooked meat medium . At the same times a piece of muscle and exudate were inoculated on the

surface of 10% sheep blood agar , then incubated anaerobically at 37°C for 48 hours . The plates were examined for characteristic colonies of *C. chauvoei, C. septicum* and *C. perfringens* type "A". Subcultures from suspected colonies in cooked meat broth were made for further biochemical identification according to (Smith and Holdeman, 1968, Koneman et al., 1992 and Collee et al., 1996).

Pathogenicity test :

A piece of infected muscle was ground with sterile physiological saline in a sterile morter . Guinea pigs were inoculated intramusculary with 0.5 ml of the triturated material mixed with 0.5 ml of 5% calcium chloride solution .Post mortem examination of the dead animals showed haemorrhagic oedema spreading from the site of infection .Then liver impression smears stained with Gram stain were examined microscopically. As well as reisolation of *C.chauvoei, C. septicum* and *C. perfringens* type "A" from liver , heart blood and the site of infection was carried out according to (Dabrowski and Cygan, 1966 and Al-Khatib, 1969) .

Animal protection test:

Guinea pigs were inoculated with *C.chauvoei, C. septicum* or *C. perfringens* type "A" antiserum (were obtained from Burroughs Welcome Research Laboratories, Beckenham, London, England) 24 hours before inoculation with the culture of the isolates of *C.chauvoei, C. septicum* or *C. perfringens* type "A", were investigated according to the method described by (Sterne and Batty, 1975 and Quinn et al., 1999).

Enzyme linked immunosorbent assay (ELISA):

ELISA was performed for the 34 isolates according to (Iacona et al., 1980). ELISA plates 96 flat bottom wells were coated each with 100 µl of 20µg soluble *C. chauvoei, C. septicum* or *C. perfringens* type "A" antigen (isolates) in carbonate buffer pH 9.6 separately. The plates were incubated overnight at room temperature. Following blocking with 0.1 % bovine serum albumin (BSA) in coating plates, 100 ul of the antitoxin (*C. chauvoei, C. septicum* or *C. perfringens* type "A") diluted at 1:100 in PBS were added and the plates were kept for 2 hours at 37°C in a shaking water bath . After washing the plates 5 times with PBS containing 0.05% tween 20, 100ul of alkaline phosphatase labeled anti- sheep IgG antibodies diluted at 1: 3000 in PBS were added and the plates were kept for 1 hour at 37°C in a shaking water bath. The chromogen paranitrophenyl phosphate, at 1mg per 5 ml substrate buffer , pH 9.8 , was added and the absorbance of the coloured reaction was read within 30 minutes at 405 nm using a titertek multishan ELISA reader. The positively threshold value (cut off value) was determined as double fold of the mean value of negative sera.

Extraction of DNA from muscles:

Bacteria in 1ml of culture were pelleted by centrifugation and used as sourse of chromosomal DNA .

To isolate DNA from (3) muscle samples of buffalo calves and sheep was performed according to the method of (Takeuchi et al., 1997 and Sasaki et al., 2001) , approximately 0.5mg of each muscle was minced and resuspended in 3 ml of phosphate buffered saline (PBS) , then centrifugated at 1000 x g for 5 minutes , at pH 7.4. The supernatant was centrifugated at 1200 x g for 10 minutes . The pellet was washed once with 1.5 ml of PBS , and resuspended in 150 ul of sucrose-TES (25% sucrose, 10mM Tris-HCL, 1mM EDTA, 150mM NaCL) containing 10mg /ml of lysosome .The sample was left for 30 minute at 37 oC before adding 10 ul of proteinase K and a further incubation for 1hour at 37°C. The samples were diluted with 150 ul of TES buffer before being extraction twice with phenol and once with phenol/ chloroform / alcohol (50/48/2). Finally precipitated with ethanol. DNA was eluated in 40 ul of TE buffer (10mM Tris-HCL, 1mM EDTA, pH 8.0).

Primers used in PCR:

Specific oligonuclotide one primers for *C. chauvoei* and *C. septicum* were selected on the presence of 71-bp insert in the 16S- 23S rDNA spacer region of *C. septicum* (Sasaki et al., 2000a), a forward primer (5`-GAAAATTGCACATGAATTAAA-3`) and 23UPCH (Sasaki et al., 2000b) was used as a reverseprimer (5`GGATCAGAACTCTAAACCTTTCT-3`) .
Amplified fragment : 522bp for *C. chauvoei* , and 594 bp for *C. septicum* .

C. perfringens type "A" primers were selected from the oligonucleotide sequence published by (Saint-Joanis et al., 1989), a forward primer (5`-AAGATTTGTAAGGCGCTT-3`) and a reverse primer was (5`-ATTTCCTGAAATCCACTC-3`), with amplified fragment: 1167bp .

PCR amplification:

The PCR was performed according to (Sasaki et al., 2000b) in a touchdown thermocycler in a total reaction volume of 50 ul containing 5 ul of 10 x PCR buffer (10 mM tris- HCL, pH 9.0, 50 mM KCL, 0.005% Tween , 0.1% Tritonx -100), 5 ul of 25 mM $MgCl_2$, 250uM each deoxynucleotide triphosphate, 2 units of Taq DNA polymerase, 1 uM of each primer and 5ul of tamplate DNA. Amplification was obtained with 35 cycles following an initial denatureting step at 94°C for 3 sec. Each cycle involved denaturation at 94°C for 1 minute, annealing at 56°C for 1 minute, and extension at 72°C for 1.5 minutes. The final extension was performed at 72°C for 7 minutes .

For the detection PCR products , a 10 ul of amplified DNA was examined by electrophoresis

0.7% agarose gel, and visualized with ethidium bromide and UV light . Electrophoresis was carried out for 2 hours at 110 V.

RESULTS

The results obtained in Table (1) and Fig. (1) , demonstrate that *C. chauvoei*, *C. septicum* and *C. perfringens* type "A" were isolated from the infected muscles and exudate of buffalo calves with incidence of 8.8% , 6.2% , and 11.3% , respectively .While the isolation rate of *C. chauvoei* , *C. septicum* and *C. perfringens* type "A" from infected muscles and exudate of sheep was 3.8%, 5% , and 7.5% , respectively .

Table (2) was illustrated the results of application of ELISA for diagnosis and differentation between 3 types of clostridia (*C. chauvoei* , *C. septicum* and *C. perfringens* type "A") .The ELISA was proved to be a reliable test with an excellent agreement with the animal inoculation and cultural methods. The optical density (O.D) range from (0.21- 0.86), avalue of 0.35 O.D units was regarded as the minimum for a positive test by ELISA , while value below 0.35 was recorded negative. For 34 isolates ELISA shows that 8 isolates (23.5%) was classified as *C. chauvoei* ,7 (20.6%) as *C. septicum* and 12 isolates (35.3%) as *C. perfringens* type "A". Seven (20.6%) isolates could not be classified.

The specificity of the oligonucleotide primer was confrimed by the positive amplification of 522 , 594 and 1167 bp fragments from the extracted DNA of *C. chauvoei* , *C. septicum* and *C. perfringens* type "A" respectively, as shown in photo (1 and 2) .

These findings suggest that PCR is a useful assay in identifying of clostridial blackleg and gas gangarene microorganisms directly from clinical material.

Table (1): Results of bacteriological examination of clostridial blackleg and gas gangarene organisms isolated from infected muscles and exudate samples of buffalo calves and sheep.

The examined animal	No.of examined samples	*Clostridium* species isolates						Total no. of positive samples	%
		C.chauvoei		*C.septicum*		*C.perfringens* type "A"			
		No.	%	No.	%	No.	%		
Buffalo calves	80	7	8.8	5	6.2	9	11.3	21	26.3
Sheep	80	3	3.8	4	5	6	7.5	13	16.3
Total	160	10	6.3	9	5.6	15	9.4	34	21.3

% was calculated according to the number of examined samples .

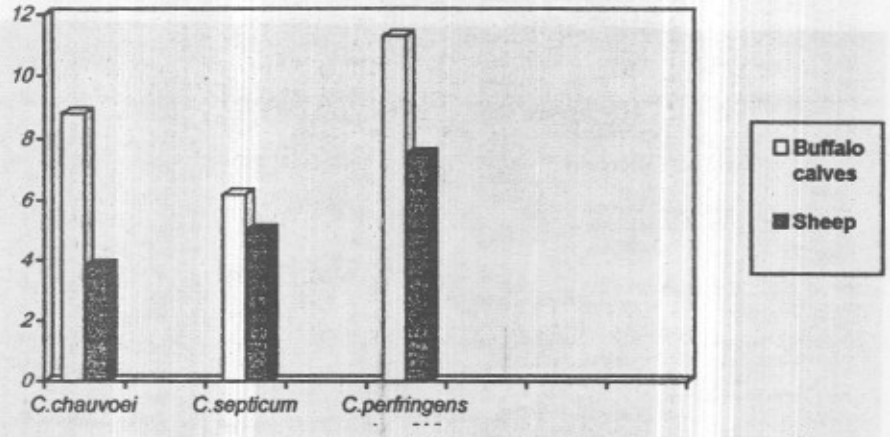

Fig.(1): Percentage of clostridial blackleg and gas gangarene organisms isolated from infected muscles and exudate samples of buffalo calves and sheep.

Table (2): Results of ELISA for diagnosis of clostridial blackleg and gas gangarene organisms isolated from infected muscles and exudate samples of buffalo calves and sheep.

Clostridium species	No. of positive samples by cultural method	ELISA			
		Positive	%*	Negative	%*
C.chauvoei	10	8	23.5	2	5.9
C.septicum	9	7	20.6	2	5.9
C.perfringens type "A"	15	12	35.3	3	8.8

%* was calculated according to the total no. of isolates (34) by cultural method.

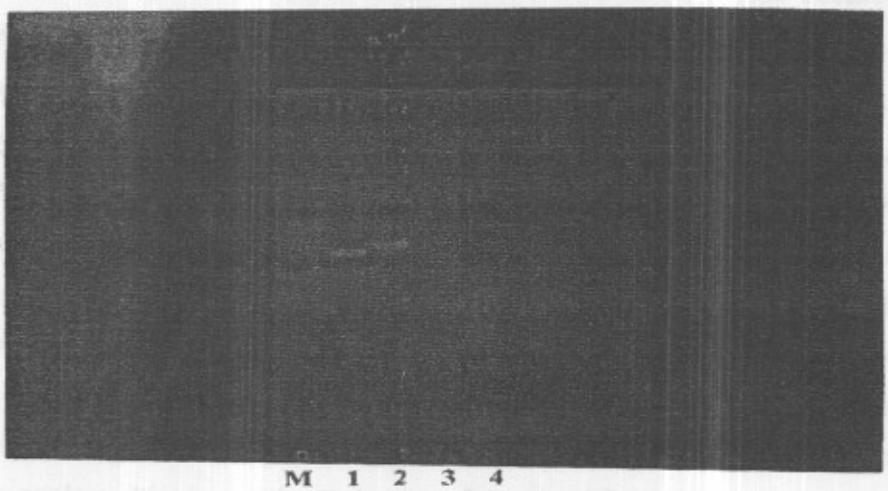

Photo (1): Electrophoresis analysis of PCR product of amplified *C. chauvoei* and *C. septicum* from infected muscles .

M : 100bp marker.

Lane 1 shows positive amplification of the 522bp fragment of *C.chauvoei* .

Lane 2 shows positive amplification of the 594bp fragment of *C. septicum*.

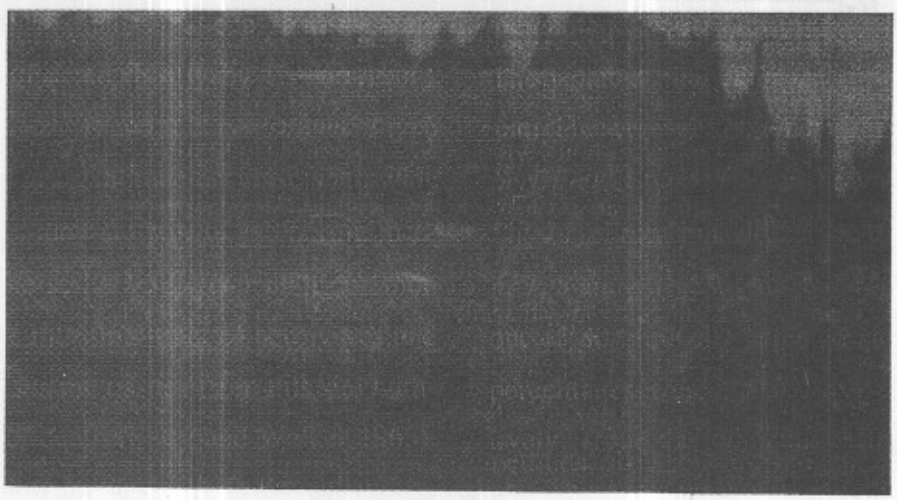

Photo (2): Electrophoresis analysis of PCR product of amplified *C. perfringens* type "A" from infected muscles .
M : 100bp marker.
Lane 3 show positive amplification of the 1167bp fragment of *C.perfringens* type "A".

DISCUSSION

Clostridial myonecrosis (gas gangrene) are economically important diseases characterised by high mortality in cattle, sheep and buffalo calves.They are caused mostly by *C. chauvoei* . *C.septicum, C. novyi* and *C. perfringens* type "A". Clostridial gas gangrene organisms are wide spread in nature, they are part of the normal bacterial flora of the soil. *C. septicum* is a common inhabitant of the intestinal tract of herbivores and apparently invades the tissues from this site within a few hours of death (Batty and Walker 1963). The differentiation between these organisms by traditional microbiological methods is difficult,because the detection and identification of these clostridia depends on clinical signs , growth on sheep blood agar, biochemical tests , neutralization of lethal toxins, and immunological methods, but all these reaction are time consuming and laborious . There is a great need for simple methods, so we established ELISA and PCR assay for the detection and identification of pathogenic clostridia in gas gangrene .

The isolated microorganisms from the examined samples were *C. chauvoei, C.septicum* and *C. perfringens* type "A" as shown in Table (1) and Fig. (1). From buffalo calves they were detected in 7, 5 and 9 (8.8%, 6.2 and 11.3%), respectively out of 80 clinically affected samples. While the percentages were 3.8% , 5% and 7.5% respectively, from the clinically affected sheep samples.These results are nearly in agreement with (Ryff and Lee, 1946 and Roberts 1959) who isolated *C. chauvoei,* either alone or together with *C.septicum* and *C. perfringens* from examined muscles from dead cattle and sheep and isolated *C.septicum* and *C. perfringens* from muscles as primary cause of gas gangrene. (Blood et

al.,1979 and Rue Jensen and Swift 1982) stated the true blackleg is caused by *C. chauvoei* , but malignant oedema caused by *C. septicum*.. Also (Malone et al., 1986) identified cases of *C. chauvoei* infection in cattle with lesions of myositis lesion of fibrinous pericarditis and lesions of purulent meningits. (Romarao and Rao 1990) reported that the annual incidence of black quarter disease caused by *C. chauvoei.* (Kuhnert et al., 1997) identifed *C. chauvoei* as aetiological agent of an outbreak of blackleg in cattle either directly from clinical samples of muscle, liver, spleen and kidney or from primary cultures of this material. During blackleg outbreak (Rajaonarison et al., 2001) isolated *C. septicum* . (Pinto et al.,2005) stated that malignant oedema is a fatal infection of skeletal muscles and subcutaneous tissue of ruminants and other animal species caused mainly by *C. septicum* but can also occur in association with *C. chauvoei* , *C. sordellii, C. novyi* and *C. perfringens* type"A".

As shown in Table (2) ,the use of enzyme linked immunosorbent assay (ELISA), recorded that out of 34 positive strains, 8 isolates (23.5%) were identified as *C. chauvoei* , 7 (20.6%) as *C. septicum* and 12 isolates (35.3%) as *C.perfringens* type "A" by using ELISA. These agreed with the results of (Poxton and Byrne 1984 and Baldassi et al., 2001) who concluded that ELISA is considered as an alternative to mouse neutralization test (MNT), for the detection of *C.perfringens* toxins and allows the differential diagnosis of clostridia species. They added that ELISA is slightly more sensitive and faster test giving results within 4 hours, while mouse neutralization test required 48 hours.

C. chauvoei and *C.septicum* are two closely related species found in a variety of pathological conditions. Both are pleomorphic and exhibit forms of great similarity. The biochemical reaction of the two organisms are also similar although they differ in the fermentation of sucrose .The only certain method of discrimination has been by protection tests in animals, but even the antiserum to *C. septicum* will sometimes give protection against challenge with *C. chauvoei* (Batty and Walker, 1963 and Smith and Holdeman, 1968).

The presence of *C. perfringens* is also a public health problem, since human can be intoxicated by ingestion of contaminated meat. *C. perfringens* has been identified by seroneutralization in laboratory animals using specific antisera. This toxin typing technique requires continuous supply of laboratory animals and use of monovalent domestic sera which are difficult to find and extremely expensive. Moreover toxin typing results are obtained in 24 or even 48 hours observation (Sterne and Batty, 1975 and Kadra et al.,1999

The use of polymerase chain reaction (PCR), shown in Photo (1), revealed positive amplification of 522bp and 594bp fragments *C.chauvoei* and *C.septicum.* While Lane 3

Photo (2) indicates positive amplification of 1167bp fragment of *C.perfringens* type "A". These results are in agreement with the results of (Havard et al., 1992; Meer and Songer,1997; Kanakara et al., 1998; Kadar et al., 1999 and Gkiourtzidis et al., (2001), who used PCR technique as a diagnostic assay for identification and typing of *C. perfringens* strains that cause enterotoxaemia in sheep. (Piatti et al., 2004) recorded that PCR is a useful assay for detection and identifcation of alpha toxin of *C. perfringens* , also is a providing a good alternative to the time consuming and specific mouse neutralization test normally used in laboratory routine .

PCR is valuable tool for rapid detection and identification of *C. chauvoei* and *C.septicum* in both cultures and tissues , which can replace the fastidious traditional identification methods and replace laboratory animals testing (Kuhnert et al., 1997; Takeuchi et al., 1997; Sasaki et al., 2000a and Uzal et al., 2003). (Sasaki et al.,2000b) found that rapid identification and differentiation of pathogenic clostridia (*C. chauvoei* , *C. septicum* , *C. novyi* and *C. sordellii*) in gas gangrene by PCR based on the 16S-23S rDNA spacer regions. Also (Sasaki et al., 2001) used PCR for detection of *C. chauvoei* and *C. septicum* in clinical specimens by single primer.

It is conculded that PCR assay is suitable and rapid diagnostic method for identification of clostridial blackleg and gas gangrene (*C.chauvoei*, *C.septicum* and *C. perfringens* type "A") from clinical specimens and provides a good alternative to the time consuming in the routine laboratory diagnosis and more specific than mouse neuteralization test and ELISA .

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تطبيق بعض الطرق الحديثة والسريعة لتشخيص مرض التفحم العضلى وغرغرينا العضلات فى عجول الجاموس والأغنام

د. إلهام إبراهيم عطوة* ، د. مرفت عبدالرحمن على**
* قسم البكتريولوچى بمعهد بحوث صحة الحيوان شبين الكوم – منوفية
** قسم البكتريولوچى بمعهد بحوث الحيوان الدقى – جيزة

يعتبر مرض التفحم العضلى وغرغرينا العضلات من أخطر الأمراض التى تصيب الجاموس والأغنام وتؤدى إلى خسائر إقتصادية كبيرة وذلك بسبب نسبة النفوق العالية.

فى هذه الدراسة تم الفحص البكتريولوچى اللاهوائى لعدد ١٦٠ عينة من عضلات مصابة والسائل الموجود بين العضلات (٨٠ عينة من عجول الجاموس و٨٠ عينة من الأغنام) تم تجميعها من مزارع مختلفة. تم عزل ميكروبات *الكلوستريديم شوفياى والكلوستريديم سيبتكم والكلوستريديم بيرفرنجينز* نوع (أ) من عجول الجاموس المصابة بنسبة ٨.٨٪ و ٦.٢٪ و ١١.٣٪ على الترتيب. كما تم عزل نفس العترات (*الكلوستريديم شوفياى والكلوستريديم سيبتكم والكلوستريديم بيرفرنجينز* نوع (أ)) من الأغنام المصابة بنسبة ٣.٨٪ و ٥٪ و٧.٥٪ على الترتيب.

تم إستخدام إختبار الأليزا على العترات المعزولة لتصنيفها بالمقارنة بإختبار الحقن التعادلى فى الفئران والحقن فى الأرانب الهندى. وقد أثبتت النتائج كفاءة وسرعة إختبار الأليزا فى تصنيف العترات كطريقة للتشخيص المعملى السريع.

وإشتملت أيضاً الدراسة على إستخدام إختبار تفاعل البلمرة المتسلسل كطريقة حديثة وسريعة لتشخيص *الكلوستريديم شوفياى والكلوستريديم سيبتكم والكلوستريديم بيرفرنجينز* نوع (أ) مباشرة من العضلات المصابة بالتفحم العضلى وغرغرينا العضلات. وقد وأثبتت النتائج سرعة ودقة إختبار تفاعل البلمرة المتسلسل فى التشخيص السريع للمرض فى خلال ساعات قليلة من بداية ظهور الأعراض. وهذه تعتبر الأكثر حساسية وأفضل وأسرع طريقة لتشخيص المرض بإستخدام بريمر واحد للتفرقة بين *الكلوستريديم شوفياى والكلوستريديم سيبتكم* وبريمر أخر لتمييز *الكلوستريديم بيرفرنجينز* نوع (أ).