BS. VET. MED. J. 7TH SCI. CONF. VOL. 22, NO.1, P.53-57

Beni-Suef Veterinary Medical Journal

Molecular Typing of Clostridium perfringens Isolates from Soil, Healthy, and Diseased Sheep in Egypt by Multiplex PCR

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In this study multiplex PCR was used for typing of *Clostridium perfringens* isolates from soil, clinically healthy and diseased sheep. *Clostridium perfringens* was isolated from 41 out of 100 soil samples, 12 out of 100 clinically healthy sheep and 118 out of 200 sheep with enterotoxaemia signs. Genotyping of 41 isolates from soil indicated that 29 (70.73%) were type A, 3 (7.31%) were type B and 9 (21.95%) were type D. Of 12 isolates from clinically healthy sheep 6 (50%) were type A and 6 (50%) were type D. Of 118 isolates from diseased sheep 42 (35.59%) were type A, 22 (18.64%) were type B and 54 (45.76%) were type D. This result indicates that *Clostridium perfringens* type A, B and D are the main types causing enterotoxaemia in sheep in Egypt and *Clostridium perfringens* type A must be included in any vaccine programme to ensure optimum protection.

Sheep enterotoxaemia was reported to be produced by *Clostridium perfringens* (Uzal and Songer 2008). All five types of *Clostridium perfringens* were incriminated as a cause of enterotoxaemia in sheep (Gkiourtzidis *et al.,* 2001). This group includes diseases as lamb dysentery, haemorrhagic enteritis, struck and pulpy kidney. On occasions type A *Clostridium Perfringens* is responsible for haemorrhagic enteritis and haemolytic disease or gas gangrene (Lewis, 2000).

Enteric *Clostridium perfringens* infections in sheep, goat and other species are generally called enterotoxaemia as because toxins produced in the intestine may be absorbed into the general circulation. The main diseases belonged to enterotoxaemia group are illustrated in (Table, 1) (Uzal and Songer 2008).

Diagnosis and typing of *Clostridium perfringens* depends on Detection of major toxins (in the gut, the blood stream, serous exudates of affected animals and in the supernatant fluids of culture). A problem that the standard assay in serum neutralization test performed in guinea pigs or mice which had become increasingly undesirable due to expense, complexity, disfavor on humanitarian ground and lack of sensitivity and specificity (Songer and Meer, 1996).

Recently PCR based DNA amplification of the genes for the toxins α, β, ε, and ί was developed for rapid and accurate typing of *Clostridium perfringens* (Daube *et al.,* 1994; Buogo *et al.,* 1995; Uzal *et al.,* 1996). Diagnosis of enterotoxaemia relies on the detection of either the toxin with bioassays or the toxin genes by PCR (Albini *et al.,* 2008). The aim of this study was to type *Clostridium perfringens* strains isolated from soil, healthy and diseased sheep by multiplex PCR.

Table (1): The main diseases belonged to enterotoxaemia group in sheep.

Clostridium perfringens types	Disease in sheep
A	Yellow lamb disease
B	Lamb dysentery and Hemorrhagic enteritis
C	Struck
D	Pulpy kidney disease
E	Enterotoxaemia (scant information available)

Material and methods

Sample collection. Eight hundred sheep were regularly visited during the period from December 2008 to November 2009 to inspect the animals for clinical signs of enterotoxaemia. Sheep were reared in flocks located at Desert road, Waedielnatron, Merkz bader and Sadat city. Four hundred (400) samples were collected from soil of sheep farms, clinically healthy and sheep with enterotoxaemia for isolation of *Clostridium perfringens*. The types and number of collected samples are illustrated in (Table 2).

Table (2): The number and types of collected sample for isolation of *Clostridium perfringens*.

Type of samples		Number
Soil samples		100
Examined suspected animals	Fecal samples	70
	Intestinal contents	130
Apparently healthy animals*	Fecal samples	50
	Intestinal contents	50
Total		400

* Samples collected from apparently healthy sheep were collected from El–Moonib slaughterhouse.

***Clostridium perfringens* isolation.** *Clostridium perfringens* was isolated according to Willis, (1977). In which One gram of the intestinal content or fecal swabs or two pea size particles of each fecal samples or soil samples were inoculated into freshly prepared boiled and cooled cooked meat medium. Purification carried out on 10% neomycin sulphate sheep blood agar. The colonies showing double zone of haemolysis were identified according to Cruickshank *et al.,* (1975); Koneman *et al.,* (1988) based on colony morphology, microscopical characters and biochemical reaction.

DNA extraction. One ml of over night culture in fluid thioglycollate broth (FTB) with D – cycloserine was centrifuged for 20 minutes at 13000 rpm. The pellets were suspended in microfuge tube containing 600 µl of ice cold cell lysis buffer, vigorously mixed then add 3 µl of proteinase K solution to the lysate to increase the yield of genomic DNA and incubated at 55 °C for 3-16 hours. The extraction process completed according to Sambrook and Russell, (2001). Finally the extracted DNA pellet stored in 100 µl of tris EDTA buffer (TE) at -20 °C for further analysis.

Polymerase chain reaction. PCR was done in rotor gene machine according to Greco *et al.,* (2005). The reactions were performed in 50 µl volumes in micro amplification tubes (PCR tubes). The reaction mixture consists of 10 µl DNA template, 5 µl of 10X Dream Taq green buffer, 3 µl of dNTPS 10mM of each, 0.5 µl of Dream Taq polymerase (2.5 u), 1 µl of each primer (50 pmoles/ µl) and 23.5 µl of DNA free water. The primers were synthesized by *Bioneer Company Germany*.

The nucleotide sequences of primers used for PCR amplification of genes for *Clostridium perfringens* toxins α, β, ε and ί were illustrated in (Table 3) according to Greco *et al.,* (2005).

The PCR was carried out with preliminary step at 95°C for 5 minutes followed by 35 cycles consisting of 1 minute of denaturation at 94°C, 1 minute of annealing at 50°C and 1 minute of extension at 72°C. After last cycle, final extension performed at 72°C for 10 minutes. The PCR products were stored in the thermal cycler at 4°C until they were collected. 10 µl of the amplified products together with 100 pb DNA ladder (Fermentas) were electrophoresed in a 1.5% agarose gel and stained with ethidium bromide. Amplified bands were visualized and photographed under UV illumination.

Statistical analysis was performed using Chi-square test (X^2) according to Hill, (1979).

Table (3): The nucleotide sequences of primers used for PCR amplification.

Toxin/gene	Oligonucleotide sequences	Fragment length (bp)
α/cpa	F 5-TGCTAATGTTACTGCCGTTGATAG-3 R 5-TGCTAATGTTACTGCCGTTGATAG-3	247
β/cpb	F 5-AACTTAACTGGATTTATGTCTTCA-3 R 5-ATAGTAGAAAAATCAGGTTGGACA-3	317
ε/etx	F 5-ATTAAAATCACAATCATTCACTTG-3 R 5-CTTGTGAAGGGACATTATGAGTAA-3	206
ί/iap	F 5-TTTTAACTAGTTCATTTCCTAGTTA-3 R 5-TTTTTGTATTCTTTTTCTCTAGGATT-3	298

Results

The morbidity, mortality and case fatality associated with *Clostridium* enterotoxaemia in sheep were illustrated in (Table 4). The percentage of *Clostridium perfringens* isolated from the soil, apparently healthy and diseased sheep were 41%, 12% and 59%, respectively (Table 5).

Molecular typing of *Clostridium perfringens* using multiplex PCR depends on detection of toxin genes; cpa gene that responsible for alpha toxin production, cpb gene that responsible for beta toxin production, etx gene that responsible for epsilon toxin production and iap gene that responsible for iota toxin production. Isolates contain only cpa gene considered as *Clostridium*

perfringens type A, isolates contain cpa, cpb and etx genes considered as *Clostridium perfringens* type B, isolates contain cpa and etx genes considered as *Clostridium perfringens* type D and isolates contain cpa and iap genes considered as *Clostridium perfringens* type E (Fig. 1).The percent of *Clostridium perfringens* type A, B and D were illustrated in (Table 6).

Table (4): The morbidity, the mortality and the case fatality rate.

No of animals examined	No of clinically diseased animals	No of dead animals	Morbidity rate	Mortality rate	Case fatality rate
800	200	130	25%	16.25%	65%

Table (5): The percentage of *Clostridium perfringens* isolated from the soil, apparently healthy and diseased sheep.

	Soil	Apparently healthy sheep	Diseased sheep
No of examined samples	100	100	200
Positive samples	41	12	118
Percentage	41%	12%	59%

Table (6): The percentage of different *clostridium perfringens* types isolated from soil, clinically healthy and diseases animal.

Isolates types / Source of isolates	Total number of isolates	Type A		Type B		Type D	
		No	%	No	%	No	%
Soil	41	29	70.73%	3	7.31 %	9	21.95%
Clinically healthy	12	6	50%	0	0 %	6	50%
Diseased	118	42	35.59%	22	18.64%	54	45.76%
Total	171	77	45.03%	25	14.61%	69	40.35%

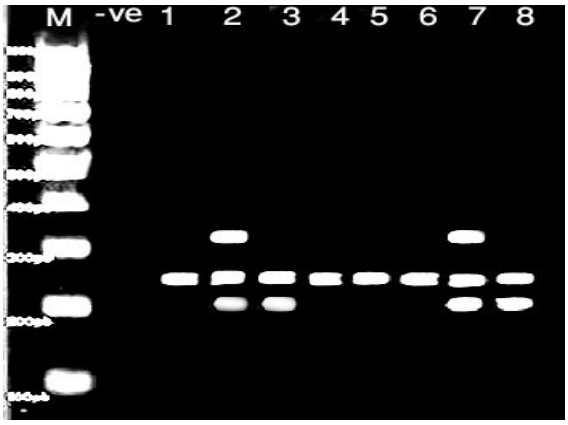

Fig. (1): Agarose gel electrophoresis of PCR products of *Clostridium perfringens* isolates.
M: 100 pb DNA ladder (Fermentas), -ve: control negative, lane 1, 2 and 3 *Clostridium perfringens* isolates from clinical cases; in lane 1 alpha toxin only (type A perferingens), in lane 2 alpha, beta and epsilon toxins (type B perferingens) and in lane 3 only toxin alpha and epsilon (type D perferingens). Lane 4 and 5 were type A and D perfringens isolated from clinically healthy animals while lane 6, 7 and 8 were type A, B and D perfringens isolated from soil.

Discussion

Clostridial enterotoxaemia of sheep caused considerable economic losses to sheep industry due to high case fatality, decreased productivity, treatment and control costs (Ozcan and Gurcay, 2000; Greco *et al.,* (2005). It ranked a third as a cause of death in sheep (Mohammad and Mohammad, 2008).

Repeated occurrence of attacks in vaccinated flocks with polyvalent vaccine directed our attention to isolation of causative bacteria with application of modern typing technique using multiplex PCR.

In this study the morbidity rate was 25%. This rate was coincided with the range of Blood and Radostits, (1989); Halil *et al.,* (2007) but higher than that reported by Osman, (1993) who reported 15.87% morbidity rate in two flocks in Upper Egypt. The mortality rate during this investigation was 16.25%. This agreed with Greco *et al.,* (2005); Halil *et al.,* (2007) but higher than mortality rates reported by Danko *et*

al., (1975); Hosli *et al.,* (1980); Sharif *et al.,* (2005) as 7.63%, 12.5% and 8.3%, respectively. The case fatality rate reported in the present study was 65%. This percent is in agreement with Mohammad and Mohammad, (2008). Findly and Butain, (1968) and Grieg, (1975) reported 50% and 10%-15%, respectively while Osman, (1993) reported 100% case fatality rate.

Agarose gel electrophoresis of the PCR products of *clostridium perfringens* using multiplex PCR were illustrated in (Fig. 1). Three types of *clostridium perfringens* were detected, *clostridium perfringens* type A, type B and type D. that ensuring the successful using of multiplex PCR in accurate and rapid typing of *clostridium perfringens* isolates. Multiplex PCR techniques have been used to determine *C. perfringens* toxin genes by some researchers Daube *et al.,* (1994); Buogo *et al.,* (1995); Uzal *et al.,* (1996); Bauns *et al.,* (2004); Albini *et al.,* (2008).

The most prevalent type of *clostridium perfringens* isolated from soil was *clostridium perfringens* type A followed by *clostridium perfringens* type D and *clostridium perfringens* type B. In accordance with our results Nagi, (1977) reported that *clostridium perfringens* type A is the predominant isolate from soil samples. *Clostridium perfringens* type A and D were equally isolated from clinically healthy sheep. Kalender *et al.,* (2005) isolated both types only from clinically healthy slaughtered sheep; while Osman, (1993) found that *clostridium perfringens* type D is the predominant isolate from clinically healthy slaughtered sheep. *Clostridium perfringens* type D was the predominant isolate in diseased sheep followed by *clostridium perfringens* type A and *clostridium perfringens* type B. This agreed with Osman, (1993); Miserez *et al.,* (1998) Who found that the predominant isolate from diseased sheep is *clostridium perfringens* type D but disagreed with Gkioutzidis *et al.,* (2001); Greco *et al.,* (2005) Who found the predominant isolates from diseased lamb are *clostridium perfringens* type B and A. This might be due to they studied clostridium enterotoxaemia among lambs only.

Conclusion

Multiplex PCR provides rapid, accurate and easy methods for diagnosis and typing of *clostridium perfringens* infection in sheep. Type A *clostridium perfringens* should be considered during vaccine manufacture in Egypt.

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