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Detection and Determination of Various Types of *Clostridium perfringens* in Sheep and Goat by Culture and ELISA Method in Khuzestan Province.

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ABSTRACT

Enterotoxaemia is one of the acute, dangerous and fatal intestinal diseases in man and domestic animals that make abundant economic losses in the livestock industry. Because of the high levels of toxins with lots of Clostridium perfringens organisms in the intestinal contents of dead or sick animals, detection of toxins and determination of various toxinotypes of Clostridium perfringens, as well as the bacteriological diagnosis of the disease and consideration of visible clinical signs and necropsy findings, is the reliable and accurate diagnostic procedure for exactly diagnosis of the disease. In present study, the samples of intestinal contents of sheep and goats, suspected to Enterotoxaemia in various cities across the Khuzestan province were evaluated by culture and ELISA methods and the results were compared. Total of 218 samples, 70 (32/1%) were diagnosed bacteriologically as Clostridium perfringens. Total 46 prepared samples tested by ELISA, among 15 (32/6%) were reacted and positive for Clostridium perfringens alpha, beta and epsilon toxins, 3 (6 /52%) were type A, 2 (4/3%) were type B, 3 (6/52%) were type C and 7 (15 /2%) were type D. In this study, the predominant type was Clostridium perfringens type D in Khuzestan province.

Keywords: *Clostridium perfringens,* toxinotype*, Elisa, bacteriologic, Khuzestan, Enterotoxaemia*

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INTRODUCTION

Enterotoxaemia is a fatal intestinal disease that affects all livestock and human. This disease is known with other names such as pulpy kidney, infectious diarrhea of neonatal lambs, enteritis Hemorrhagic and struck that belong to the major known diseases to arise from different types of Clostridium perfringens (including B , C, and D type) in sheep and goats. It is one of the common diseases with high prevalence worldwide. However, few published reports of outbreaks, especially in Iran [7]. The bacterial agent of the disease (from Clostridia family) could be surviving in the soil as heat resistant spores, and naturally in the soil and in the gastrointestinal tract of animals and humans. The bacteria proliferated under certain conditions and can be sicken animals by production of the various toxins [12 ,13]. More animal diseases caused by Clostridium perfringens are as intestinal diseases and include B, C and D types. Because of a lot amount of toxins together by many cells of Clostridium perfringens are visible in the intestinal contents of sick or dead animals, so the only way for differential diagnosis of Enterotoxaemia is capable by observation of alpha toxin in the small intestine contents [7].

Literature Reviews

Bacteriological and biochemical properties of Clostridiums

The Clostridia are most important genus of anaerobic bacteria that have medical and veterinary importance. 118 species are known so far to this time, that about 15 species can cause infection widely among animals and human. These bacteria live in the soil, water and intestinal tract of human and animals. If living conditions and growth of the organisms prepared in the body of sensitive animal and human, these bacteria quickly proliferated, and cause fatal diseases by release of deadly toxins. Clostridium perfringens is Gram-positive, rod-shaped anaerobic bacteria that have capsules, and the ability to produce heat resistant spores under improper environmental conditions. This bacteria can growth well in Laboratory culture media, especially blood agar medium, and under anaerobic conditions can produce the slightly raised, round and grayish colonies with beta hemolysis (complete hemolysis) in culture media. Clostridium perfringens is classified in 5 toxin groups (A, B, C, D, and E) [8]. Clostridium perfringens is secreted 12 different types of toxin, but their classification is based on the ability to produce 4 major toxins as alpha, beta, epsilon and iota toxin by the organism. These toxins are fatal, hemolytic and necrotic. Some other toxins (gamma, delta, kappa, lambda, etc.) involved in the destruction of human and animal cells. Each toxinotype is related to the certain intestinal infections in animals. Alpha toxin is produce by all toxinotypes, Beta toxin is produced by C and B types, epsilon toxin is produced by D and B types and iota toxin is produced by E type. Alpha toxin is coded by *cpa* gene, Beta toxin is coded by *cpb* gene, epsilon toxin is coded by *etx* gene and iota toxin is coded by *iap* gene [10].

Diagnosis

Enterotoxaemia diagnosis is generally based on clinical signs, bacteriological identification and necropsy findings. But to confirm the disease, in addition to the above, the type and infection mode of disease shall be determined by the toxinotyping method.

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Most of the tests currently used to detect toxins, one of them is “mouse protection test” that is laborious, costly, and time consuming test. In addition, the use of laboratory animals as living beings and inanimate, is the non-emotional and inhumane manner and there is a need to alternative test methods. In other hand, several serological and molecular methods are used for the detection of bacterial toxins such Counter Immuno Electrophoresis (CIE) (Henderson , 1984), Elisa (Weddell and Worthington - 1984 ; Naylor et al.-1987), Latex Agglutination test (LAT), PCR and etc. Among these methods, ELISA and LAT are used to detection of Enterotoxaemia particularly in prevalent cases of sudden death of sheep. These methods are simpler and less costly and give good quantitative results. ELISA benefits include: more sensitivity, low cost and durability of testing materials, the possibility of multiple samples simultaneously, the availability and cheap equipment, no risk of radioactive exposure and finally, is the possibility to change it into an automatic technique by use of progressive equipment [5].

MATERIAL AND METHODS

Intestinal samples

Total 218 intestinal samples were collected from sheep and goats with suspected enterotoxemia belonged to 12 different cities of Khuzestan province.

Bacteriologic procedure

All of the samples were transferred to the laboratory of Ahwaz Razi Institute and immediately processed as described below or kept in 4-6° C for no longer than 24 h before processing.

A part of samples were cultured in Thioglycolate media at laboratory and incubated at 37 ° C for three days. The remaining samples were placed and kept in at - 21 ° C untile use for ELISA test. After maximum of 72 hours, the samples were sub-cultured into blood agar (BA) media containing the antibiotic Neomycin .Cultured plates were placed in Anoxomat anaerobic jar (MART), and were incubated at 37 ° C.

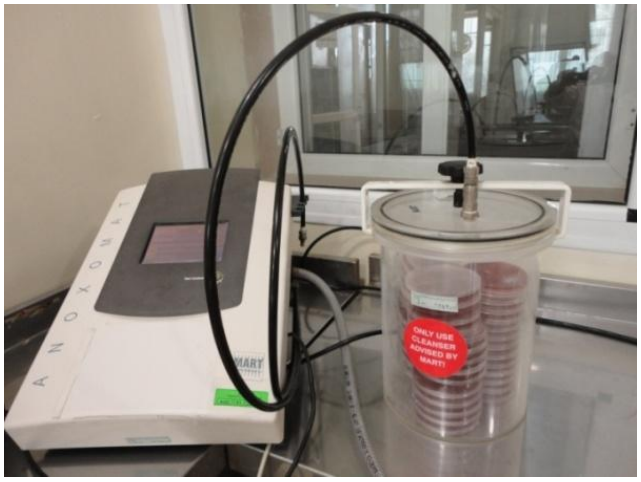

Figure 1: Anoxomat anaerobic jar (MART)

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The cultures in BA media were survey after 2 -3 next days, and suspected colonies to Clostridium (highlights, gray and opaque Colonies with beta hemolysis) were selected and prepared smear from them. The slides were examined by microscope (Clostridium bacteria are gram-positive and rod-shaped). The colonies also grown in BA were sub-cultured in different sugars for determination of biochemical properties, including: Urea, Sucrose, Salicin, Lactose, Maltose, Dextrose, Indole, Nitrate, Gelatin and Litmus milk. After the reaction, colonies were determined as Clostridium perfringens according to the following table.

type	Litmus milk	motility	Gelatin	Nitrate	Indole	Acid production					Urease
						Dextros	Maltos	Lactose	Salicin	Sucrose	
Clostridium perfringens	ACGS	-	+	+	-	+	+	+	V	+	-

A: acid C: clot G: gas S: stormy V: variable

Table 1: biochemical properties of Clostridium perfringens

Elisa procedure

The presence of C. perfringens toxins in the supernatants was determined by an indirect ELISA commercial kit (Diagnostics Cypress - Belgium), according to the manufacturer's instructions. In the test, rows A, C, E, and G were sensitized with the specific antibodies for alpha, beta, or epsilon toxin, while rows B, D, F, and H contained non-specific antibodies.

The samples, positive control and negative control scheme is as follows:

	1	2	3	4	5	6	7	8	9	10	11	12
A	P	S3										
B	P	S3										
C	N	S4										
D	N	S4										
E	S1	S5										
F	S1	S5										
G	S2	S6										
H	S2	S6										

P: positive control N: negative control S1: sample No.1 S2: sample No.2

Table 2: The schematic situation of samples in the plate of Elisa

The following steps, according to the Kit manufacturer's instructions were performed for Elisa test:

- Before use, all reagents and solutions were placed at room temperature (18-24°C).

- Washing solution was diluted by Reverse Osmosis (RO) water to the proportion of one to 20 (Accuracy conceptual framework before it is all gone crystals).

- Once again, the diluted washing solution more diluted 5 times.

- The sample was diluents as same volume by dilution buffer kit set. Briefly, 100 µl of test sample, and negative and positive controls were added to the appropriate wells and the plates were then incubated at room temperature for 1 h. After this first incubation the plates were washed 3 times and 100 µl of conjugate solution (1:50) was added into each well and then incubated at room temperature for 1 h(while keep away from light). After this incubation and washing of the plates, 100 µl of indicator solution (TMB- a mixture of chromogen and substrate) was added to each well. All the plates were then incubated at room temperature for 20 min. After this incubation, reaction was stopped by adding 50 µl of stop solution (1 M phosphoric acid). Finally, the optical densities (OD) were recorded at 450 nm using a micro plate reader (BIO-TEK, ELx800).

The net OD for each sample was calculated by subtracting the OD of the corresponding negative control from the reading of each sample well. According to the manufacturer QC data sheet, the limit of OD positivity for the alpha, beta, and epsilon toxins is 0.150; therefore, any sample that yielded a difference in OD ≥ 0.150 was considered positive for the toxins tested. Conversely, any sample that yielded a difference in the OD < 0.150 was considered negative.

The results were reviewed and summarized base on the toxinogenic characterization of Clostridia and reference model provided by the kit manufacturer were as follows:

Toxin / Toxinotype	alpha	beta	epsilon	iota
A	++	-	-	-
B	+	++	+	-
C	+	++	-	-
D	+	-	++	-
E	+	-	-	+

Table 3: Results based on the toxinogenic characterization of Clostridia

RESULTS

In the present study, total 218 intestinal content samples collected from the sheep and goats in various cities of Khuzestan province. After bacteriological culture, 70 (32/1%)

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were eligible properties of Clostridium perfringens they were confirmed via colony formation on BA contained antibiotic and biochemical properties.

Figure 2: Colony characteristics and beta hemolysis on BA

46 samples also were tested by ELISA and the following results were obtained:

Type of Clostridium perfringens	D	C	B	A	Total
Total positive reactions in Elisa	7(%15/2)	3(%6/52)	2(%4/3)	3(%6/52)	15(%32/6)

Table 4: Number and contamination rate of Clostridium perfringens biotypes in sheep and goats intestinal contents of 46 samples were suspected to enterotoxaemia that detected using ELISA

15 cases out of 46 (32/6%) that tested by ELISA were contain different toxins of Clostridium perfringens among them, type D, has the highest frequency. Bacteriologic and Elisa results are as follows:

Table 5: Bacteriologic and Elisa results for Clostridium perfringens

city	Shushtar Arab Hassan	Shushtar Arab Asad	Ramhormoz	Sussangerd	Ahwaz (Albaji)	Hamidieh	Mahshahr	Ramshir	Haftkel	Bagmalek	Total
Total samples	**20**	**35**	**23**	**10**	**29**	**10**	**14**	**30**	**15**	**32**	**218**
Bacteriologic results (Clostridium perfringens)	**6**	**12**	**10**	**3**	**9**	**3**	**4**	**9**	**3**	**11**	**70(%32/1)**
Elisa results -Positive toxins (of 46 samples)	**-**	**6**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**9**	**15(%32/6)**

DISCUSSION

Enterotoxaemia is an especial disease and is more prevalent than other diseases among the animals that grazing on the pasture or moving long distances. This disease makes abundant economic losses to the sheep keeping industry [7, 9].

The results obtained in the present study, shows the percentage of contamination with conventional bacteriological method and ELISA, a sensitive method for detection of Clostridium perfringens.

It have been reported that ELISA is a convenient , exact and cost-effective method for the diagnosis of Enterotoxaemia when incidence of sudden death in sheep for confirmation of bacteriological diagnosis and detection of toxins in the intestinal contents of animals suspected to Enterotoxaemia is acceptable and valid to 95 percent [7].The cause of positive reaction in Elisa test is presence of low amount of toxins in intestinal contents and detection of this low amount of toxin, express presence of enterotoxaemia disease in herds. Reports submitted by researchers worldwide mentioned on contamination rate between 24/3% to 100% and in these studies, type A was the dominant type among the suspected Enterotoxaemia cases in sheep [9]. The occurrence of Enterotoxaemia disease that reported in Turkey was between 38/63% to 50% and type D was the dominant type in these pollutions. Detection of high incidence and prevalence of type A in the Enterotoxaemia cases or suspected cases is that the disease at first started with Type A and then type A is capable converted to type D and just by Molecular approaches to the diagnosis, can exactly detected of them, but in conventional bacteriological, Elisa and other tests, What is diagnosed, is the type D. These results are consistent with studies by molecular method in Turkey [7,11,12]. In present study , the cases suspected to Enterotoxemia have been diagnosed in the prepared samples from sheep on northern cities of Khuzestan province especially cities of Bagmalek ,Haftkel , Ramhormoz and Shushtar that are the areas for more movement and the site of settle of nomadic livestock from neighbor provinces.If the vaccination program against Enterotoxaemia diseases not done correctly in animals of such areas , Enterotoxaemia possible outbreaks and occur more economic losses due to death of livestock. Unfortunately, in many areas, vaccination is not done or only one dose of vaccine injection will suffice. While, it should be administered two doses of vaccine with two weeks apart in the spring and fall. So It's one of the reasons for the presence of Clostridium between livestock and occurrence of Enterotoxaemia is poor immunity of animal mothers and don't transfer enough immunity to newborns and also due to the stresses caused by transportation of livestock during long movement, that are predisposing factors for changes in diet and occurrence of Enterotoxaemia disease in livestock.Hamad *et.al* (2010)recommended that a poor vaccination schedule against Enterotoxaemia should be implemented for sheep flocks and provide adequae protective immunity against all types of C. Perfringens specially types A and D [9]. Diagnosis of Enterotoxaemia or suspected cases of the disease is possible by the consideration of visible clinical signs and autopsy finding , as well as bacteriological methods ,but the definite diagnosis and Confirmation of the disease should be based on the detection and differentiation of toxins in prepared samples [6] . The important point for Enterotoxaemia disease is that clinical signs are not visible during the incidence of disease and despite presence of the pollution, any symptom is not observed until the death of animal. Sometimes animals will die suddenly while grazing on pasture. Therefore, in areas with the last prevalence of the disease in prior to the change in diet, of all animals must be considered as suspected livestock [8]. In addition to the differential diagnosis of infection, using of ELISA confirmed the bacteriological methods through the determination of Clostridium perfringens biotypes, because the determination of the number and type of bacteria as well as the type of toxins found in the intestinal contents is effective in final diagnosis of disease. El Idrissi and Ward reported that compared with ELISA

and conventional methods (like test in mice or culture of microorganism), ELISA have 90/5% and 89/2% sensitivity and specificity for beta toxin and 97/4% and 94/6% for epsilon toxin respectively [6]. Vaikosen and Ikhatua reported that C and D types of Clostridium perfringens and especially D type are the main cause of occurrence of Enterotoxaemia disease in Nigeria. They reported that 91 of the 342 stool specimens tested were positive lecithin and they were confirmed as C and D type by ELISA method [6]. Measurement of toxins in the intestinal contents is the most important factor for diagnosis of Enterotoxaemia. Gokce and colleagues have reported that Clostridium perfringens type A and type D were predominant types detected in the ELISA for suspected Enterotoxaemia cases in sheep, While the type E has not been diagnosed in Enterotoxaemia outbreaks in Turkey, and type E is not involved in any outbreaks of Enterotoxaemia in Turkey [7] . As mentioned in another References, the results of our study indicated that C.perfringens type A, B, C, and D may cause enterotoxemia,but type A and D were the predominant type and major causative agents of enterotoxemia in the sheep and goats in khuzestan provience.

Suggestions

- According to the confirmation of infection in clinically normal animals in this study, it is recommended that the herd had continuous health monitoring especially in healthy animals, with a fast and accurate laboratory methods.
- Put the control diet and avoid sudden changes in animal's diet.
- To avoid any stress to the animals coming in.
- Avoid of overeating in animals, especially at the beginning of spring and autumn.
- Rapid changes of grassland and pasture if the prevalence losses.
- The proper and sanitary burial of the carcasses in the deep of Earth and using the lime juice.
- Proper and scheduled vaccination of livestock according to recommendation of Razi Institute experts.
- Further studies to identify more accurately and faster and more convenient, using the molecular diagnostic methods

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