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Detection and Characteristics of Alphatoxin Strain Clostridium Perfringens Genetic Variability, Isolated from Kazakhstan Saiga In 2010-2013.

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ABSTRACT

The clostridial bacteria emerged from dead saiga in 2010 - 2013 were identified on the basis of 16S rRNA gene as perfringens species and by the presence of plc gene, encoding only alpha-toxin and were classified as type A. The genetic variation of Clostridium perfringens alpha-toxin strains emerged from saiga was characterized in 2010 - 2013. N- and C- domains of Clostridium perfringens alpha-toxin strain structure, emerged from the saiga in 2010 - 2012 showed a very high conservatism. The structure of Clostridium perfringens alpha-toxin strain, 2013 revealed previously unknown mutations - 4 amino acid substitutions: Ala13Thr (threonine → alanine), Val47Ile (valine, isoleucine →), Ala202Asp (alanine → aspartic acid), Thr205Ala (threonine → Alanine) in the N-domain of alpha-toxin and 1 replacement of Ser363Pro (serine → proline) in the C-domain. We may assume that this mutation affects the reduction of alpha-toxin toxicity and as a result, causes Clostridium perfringens pathogenicity reduction. The degree of identity in the N-terminal catalytic domain of alpha-toxin reaches 98.4% and in C domain responsible for the binding to membranes the identity makes 99.2%. Plc gene sequence of the strains Clostridium / Saigas / 2010 / ZKO / KZ, Clostridium / Saigas / 2011 / ZKO / KZ, Clostridium / Saigas / 2012 / Kostanay / KZ and Clostridium / Saigas / 2013 / Kostanay / KZ are deposited in the GenBankdatbase under the numbers KP143658, KP143659, KP143660, KP143661, respectively.

Keywords: Clostridium perfringens,saiga, plcgene, alpha-toxin

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INTRODUCTION

One of the main protected animals of the Kazakhstan steppe zone is the saiga antelope (Saigatatarica) - a unique species of the wild steppe antelope. The world conservation union (WCU) classified this species in 2002 in its Red List as an "endangered" one.

The territory of Kazakhstan includes the main part of saiga population modern habitat. During the last ten years their number reduced to a minimum. The reason for the saiga population disappearance is not only the impact of environmental factors, but also the periodic occurrence of infectious diseases [1]. So, the mass death of saiga antelopes was registered in the Republic of Kazakhstan for three years. 12,000 heads died within the saiga habitats, 500 heads died in 2011, 1000 heads died in 2012, and 10 animals died in 2013 (Figure 1).

Figure 1: Schematic map of saiga deaths in 2010-2013

• 1 - The death of saigas in West Kazakhstan region during 2010 (12,000 heads); 2 - The death of saiga in the West Kazakhstan region during 2011 (500 heads); 3 - The death of saiga antelopes in Kostanaiskaya oblast during 2012 (1000 heads); 4 - The death of saiga antelopes in Kostanaiskaya oblast during 2013 (~ 10 headss)

In 2010-2013 the staff of the Research Institute for Biological Safety Problems (RIBSP) revealed a dead saiga pathogen which was differentiated as Clostridium rerfringens [2]. Clostridium rerfringens, anaerobic gram-positive bacterium, is an important pathogen of a man and many animal species [3]. The pathogenicity of Clostridium perfringens is conditioned by the release of various extracellular enzymes and toxins into the environment. The exact mechanism of Clostridium perfringens pathogenesis is poorly understood [4, 5]. As is well known, a widely distributed pathogen Clostridium perfringens is represented by five (A, B, C, D and E) types under classification. This pathogen synthesizes four major toxins: alpha (α), beta (β), epsilon (ε) and iota (ι), encoded by genes plc, cpb, etx, iap/ibp, respectively [6]. Alpha-toxin is considered the main one among them, as it is generated enough in all five types, and plays a key role at the occurrence of histotoxic and intestinal disease [7], and also serves as the symbol of the deadly clostridialtoxinemia [8, 9].

Based on the abovementioned facts the aim of this work is the molecular detection of Clostridium perfringens bacterium strains, emerged from the saiga in the Republic of Kazakhstan, the evaluation of the gene plc genetic variation, encoding alpha-toxin.

MATERIALS AND METHODS OF STUDY

Bacterial strains

During the period from 2010 to 2013 the saiga population inhabiting the territory of the Republic of Kazakhstan revealed 4 strains of the bacterium Clostridium perfringens. The strains were isolated from internal organs and the blood of dead saiga.

The strain Clostridium / Saigas / 2010 / ZKO / KZ is isolated in from saiga liver in 2010.

The strain of Clostridium / Saigas / 2011 / ZKO / KZ is isolated in 2011 from the blood of saiga. Clostridium perfringens strains of 2010 and 2011 are isolated near the village of Borsy in Zhanibeksky district of West Kazakhstan region, the Republic of Kazakhstan.

The strain of Clostridium perfringens / Saigas / 2012 / Kostanay / KZ is isolated in 2012 from Saiga kidney.

The strain of Clostridium perfringens / Saigas / 2013 / Kostanay / KZ is isolated in 2013 from the blood of saiga. Clostridium perfringens strains of 2012 and 2013 are isolated in Zhangeldinsky area of Kostanai region, the Republic of Kazakhstan.

The studies on the isolation of strains were carried out in the framework of the national program "Epizootological monitoring of infectious disease circulation in the saiga population inhabiting the territory of the Republic of Kazakhstan and the development of prevention methods during the years 2012-2014".

Microbiological methods of research

The isolation and cultivation of Clostridium perfringens was carried out in a Kitt-Tarozzi medium and glucose blood agar [10]. The biochemical properties of isolated cultures was studied in semisolid medium (MPPB) supplemented with 0.5% of corresponding carbohydrate and Andrade indicator. Proteolytic activity was assessed by the ability to coagulate and peptonize milk, liquefy gelatin and develop indole. The hemolytic properties of isolated cultures were studied by their growth in anaerobical conditions on glucose-blood agar [11].

The isolation of bacterial DNA

DNA isolation was performed using the commercial kit "PrepMan Ultra Sample Preparation Reagent" of "Applied Biosystems" company, according to manufacturer's protocol.

Ribotyping and typing

All strains of Clostridium perfringens were investigated in PCR to identify the genes encoding the toxins α, b, ε, ι and 16S, according to Uzal, F.A.et.all., El-JakeeJ.et.all [12, 13]. During the ribotyping and typing of Clostridium bacteria oligonucleotide primers were used on 16S gene and α, b, ε, and ι toxins (Table 1).

Table 1: Oligonucleotide primers for PCR amplification of bacteria Clostridium perfringens genes

	Primers	Sequence	Product size, b.p.	Reference
	16S	5'-AAAGGAAGATTAATACCGCATAA-3'	722	Uz al, 2010
		5'-ATCTTGCGACCGTACTCCCC-3'		
	Alpha toxin	5'-GTTGATAGCGCAGGACATGTTAAG-3'	402	El-Jakee, 2010
		5'-CATGTAGTCATCTGTTCCAGCATC-3'		
	Beta toxin	5'-AAGAAGTTTTTTTATGAAG-3'	1025	Uz al, 2010
		5'-TCTAAATAAGCTGTTACTTTGT-3'		
	Epsilon toxin	5'TACTCATACTGTGGGAACTTCGATACAAGC-3'	403	Uz al, 2010
		5'-CTCATCTCCCATAACTGCACTATAATTTCC-3'		
	Iota toxin	5'-TTTTAACTAGTTCATTTCCTAGTTA-3'	298	Uz al, 2010
		5'-TTTTTGTATTCTTTTTCTCTAGATT-3'		

PCR product of plc gene operation time

For the genetic characterization of plc gene we designed oligonucleotide primers with plc-f-1 sequence (5'-GGC AAG CTT AGC TCC ATC TC-3 ') and plc-r-2216 (5'-CCA GCT AGG CCT AAT CCT GAA A- 3'). The chemical

synthesis of primers was performed on an automated synthesizer EXPEDITE 8909 of the company Applied Biosystems.

The reaction mixture was prepared according to the protocol of «AccuPrimeTaq DNA Polymerase High Fidelity» set: 2,5µl - 10X PCR buffer, 1 µl of each primer (20 pmol / µl), 0,2 µl (1,0 U) - Taq DNA Polymerase, 4 µl - DNA, 16,3 µl - H_2O. The amplification conditions: 94ºC - 2 min; 35 cycles at 94ºC - 30 seconds, 55ºC - 30 seconds, 68 ºC - 1 min and 68ºC - 10 min. The amplification of DNA fragments for bacteria Clostridium perfringens genes was performed on a thermocycler «GeneAmp PCR 9700" of "Applied Biosystems" company. The detection of PCR products was performed on 2% agarose gel in 1 x TAE buffer with bromide ethidium. The electrophoresis of DNA amplification products was carried out by the device for horizontal electrophoresis "G-100" of "Pharmacia" company.

DNA Sequencing

DNA sequencing was performed by using dideoxysequenation method using the terminating dideooxynucleotide (Sanger method) on an automated 16-capillary sequencer Genetic Analyser 3130 xl, Applied Biosystems. POP-7 was used as the polymer of capillaries. The operating time of terminating DNA products was performed by cycle sequencing method.

The registration numbers of the nucleotide sequences. Plc gene sequence of the strains Clostridium / Saigas / 2010 / ZKO / KZ, Clostridium / Saigas / 2011 / ZKO / KZ, Clostridium / Saigas / 2012 / Kostanay / KZ and Clostridium / Saigas / 2013 / Kostanay / KZ are deposited in the database GenBank under the numbers KP143658, KP143659, KP143660, KP143661, respectively.

Phylogenetic analysis

The phylogenetic tree was developed using the program Mega 5 accrding to the results of comparative analysis for the nucleotide sequences of 16S rRNA and plc genes and Clostridium bacteria. The comparative analysis of the amino acid sequences for the alpha-toxin structure of Kazakhstan isolates with GenBank database strains was performed using the computer program VectorNTISuite 9.

RESULTS

The phenotypic characterization of the strain is not sufficient to determine a taxonomic status of the organism under study. A phylogenetic analysis of the nucleotide sequences of the 16S rRNA gene using the strains belonging to Clostridium species, deposited at the international database GenBank was performed to clarify the type perfringens of Clostridium bacteria, emerged from the saiga in 2010 - 2013.

The results of phylogenetic analysis for the nucleotide sequences of the 16S rRNA gene of Clostridium bacteria, are shown by the phylogenetic tree (Figure 2), which was developed by the program Mega 5, using the Neighbor-Joining cluster method of genetic distance calculation and bootstrap analysis, reflecting the accuracy of clustering.

The results of phylogenetic analysis showed that Kazakhstan strains of Clostridium bacteria, isolated from the saiga in 2010 - 2013 refer to Clostridium bacteria of perfringens species, since the studied strains are on the same phylogenetic branch with Clostridium perfringens strains 13 (BA000016), Clostridium perfringens ATCC 13124 (SR000246), Clostridium perfringens AN 4744102 (DQ196132), Clostridium perfringens SG7 (FJ215324), Clostridium perfringens E108 (JX267121), Clostridium perfringens JI1 (FJ215347), deposited in the GenBank database (Figure 2).

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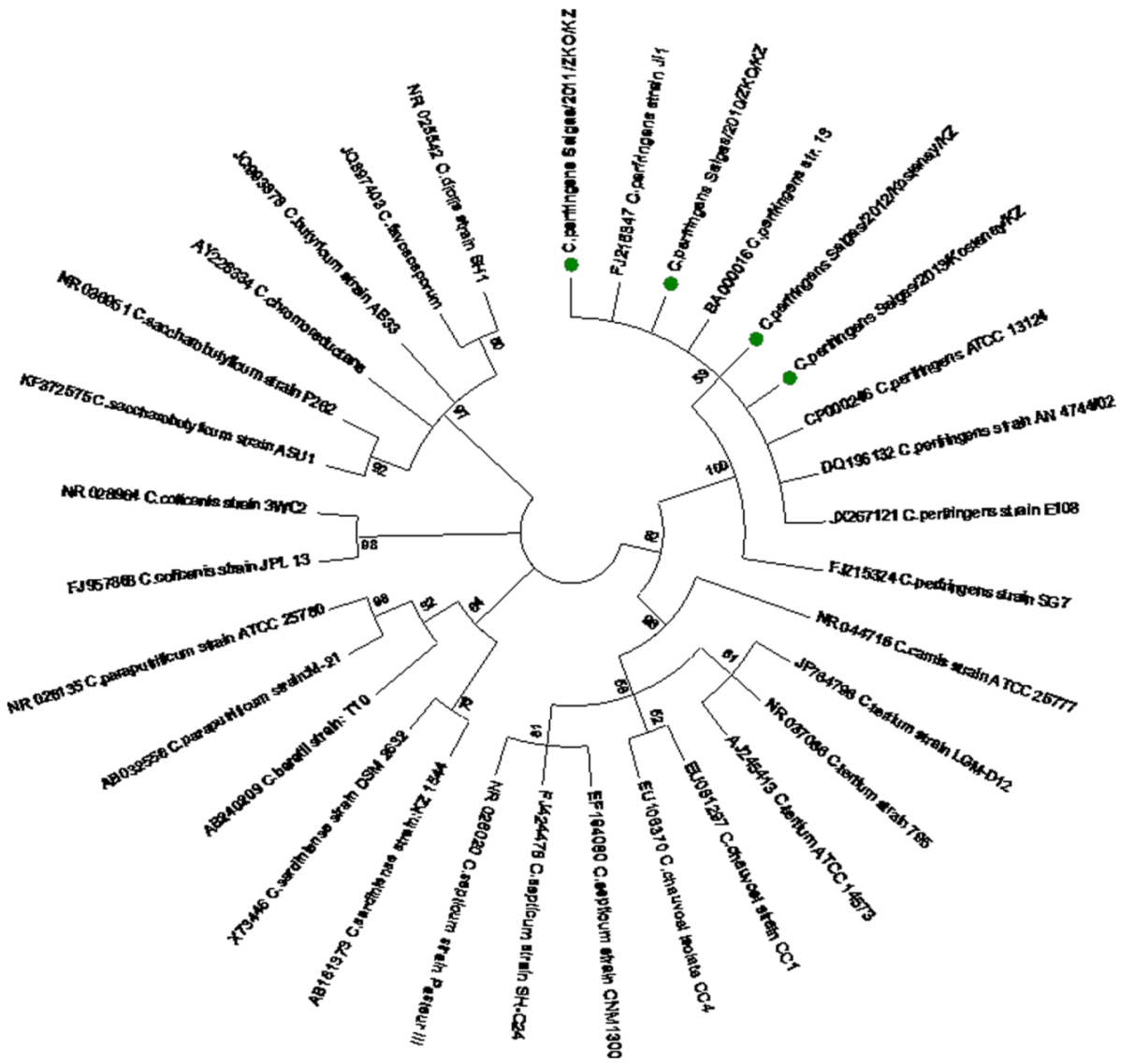

Figure 2: The phylogenetic tree developed using the bootstrap-analysis according to the comparative analysis results of the nucleotide sequences concerning 16S rRNA gene of Clostridium bacteria representatives.

According to the comparative analysis data the level of genetic relatedness for 16S rRNA gene of the strains under study with Clostridium perfringens strains from GenBank made 99%. The genetic typing of Clostridium perfringens isolates was performed by PCR analysis using the primers for α, β, ε and ι toxins [14]. All four strains Clostridium / Saigas / 2010 / ZKO / KZ, Clostridium / Saigas / 2011 / ZKO / KZ, Clostridium / Saigas / 2012 / Kostanay / KZ and Clostridium / Saigas / 2013 / Kostanay / KZ were negative for β, ε and ι toxins and positive only for the alpha-toxin (plc), thus they are attributed to the type A.

The results of the PCR amplification products concerning plc gene of Clostridium perfringens strains, isolated from the saiga in 2010 - 2013 are shown by Figure 3.

ISSN: 0975-8585

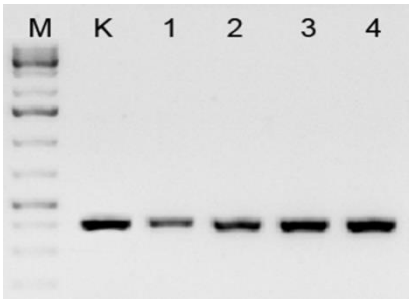

Figure 3: PCRproductsofplcgeneofClostridium perfringensstrains, emerged fromsaiga in 2010-2013

M - marker 1 kb, Fermentas; K - positive control (Clostridium perfringens, strain ATCC 13124); 1 - Clostridium / Saigas / 2010 / ZKO / KZ; 2 - Clostridium / Saigas / 2011 / ZKO / KZ; 3 - Clostridium / Saigas / 2012 / Kostanay / KZ; 4 - Clostridium / Saigas / 2013 / Kostanay / KZ

In order to determine the nucleotide sequence of the plc gene encoding the alpha-toxin conducted the sequencing Clostridium perfringens bacteria strains, isolated in 2010 - 2013 was performed. The analysis of alpha-toxin structure is carried out using the computer program Vector NTI Suite 9. Figure 4 shows the results of the comparative analysis for the amino acid sequence of the alpha-toxin structure concerning the bacterium Clostridium perfringens strains, emerged from the saiga during the period of 2010-2013 with GenBank data.

Figure 4: Comparative analysis of alpha-toxin structure in the strains of Clostridium perfringens, isolated from the saiga during 2010-2013 with GenBank data

The strains of Clostridium / Saigas / 2010 / ZKO / KZ, Clostridium / Saigas / 2011 / ZKO / KZ, Clostridium / Saigas / 2012 / Kostanay / KZ, revealed respectively in 2010 - 2012 are genetically identical (100% identity), and also with the strains of an international database GenBank: Clostridium perfringens ATCC13124 (CP000246.1), Clostridium perfringens NRRLB-23782 (DQ184137) and Clostridium perfringens NRRLB-41055 (DQ184174.1) according to plc gene, encoding alpha-toxin.

The amino acid sequence of alpha-toxin structure in the strain Clostridium / Saigas / 2013 / Kostanay / KZ, revealed in 2013, differs from the amino acid sequences of previously identified Clostridium perfringens strains (2010-2012) by 6 amino acid substitutions - Ala13Thr (alanine → threonine), Val47Ile (valine → isoleucine), Ala202Asp (alanine → aspartic acid), Thr205Ala (threonine → alanine), Ser363Pro (serine → proline), Ile373Val (isoleucine → valine).

ISSN: 0975-8585

The data of alpha-toxin comparative phylogenetic analysis concerning Clostridium perfringens strains revealed in Kazakhstan with the strains of the international GenBank database are shown by Figure 5.

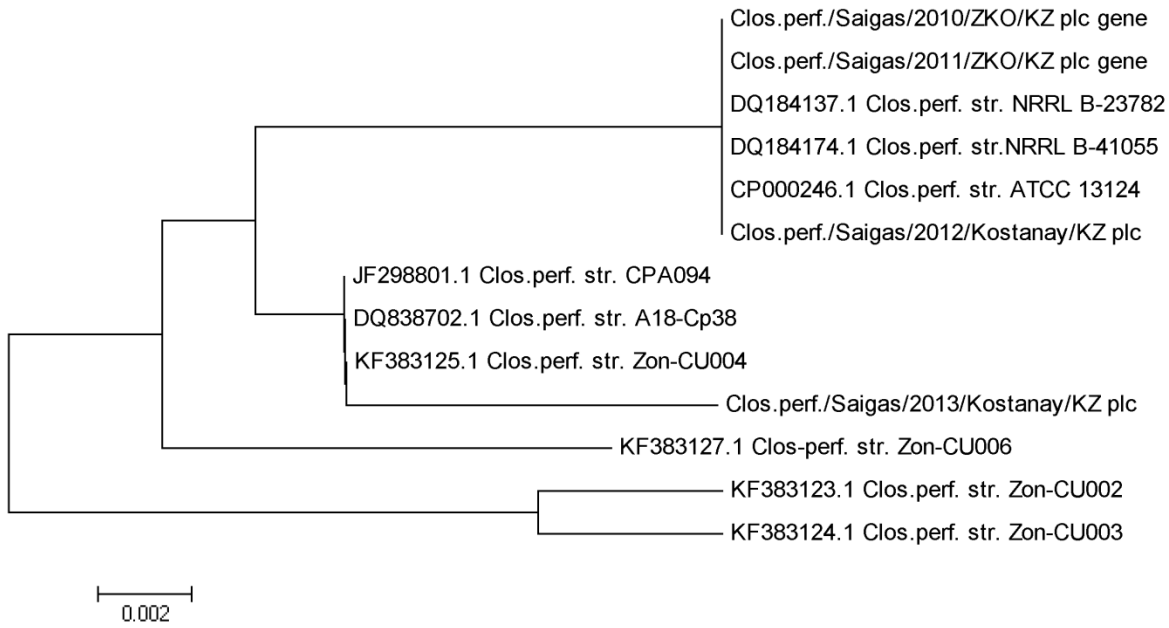

Figure 5: The phylogenetic tree constructed using the bootstrap-analysis according to the results of alpha-toxin comparative analysis in the bacterium Clostridium perfringens

Figure 5 shows, that the investigated Kazakh strains of Clostridium / Saigas / 2010 / ZKO / KZ, Clostridium / Saigas / 2011 / ZKO / KZ, Clostridium / Saigas / 2012 / Kostanai / KZ, isolated from the saiga in 2010 - 2012 respectively are closely linked to each other developing a monophyletic group and are phylogenetically related to the strains of Clostridium perfringens NRRLB-41055 (DQ184174), Clostridium perfringens NRRLB-23782 (DQ184137) from an international database GenBank.

The strain Clostridium / Saigas / 2013 / Kostanay / KZ, revealed in 2013, is located on the other branch of the phylogenetic tree and is close to the strains of Clostridium perfringens CPA094 (JF298801) and Clostridium perfringens A18-Sp38 (DQ838702) from an international database GenBank.

DISCUSSION

The mass death of saiga antelopes of West Kazakhstan in 2010 - 2011 and Kostanay region of Kazakhstan in 2012-2013 attracted the attention of public and led to the adoption of urgent and specific scientific measures for the conservation of this species, which is on the verge of extinction. Research has shown that anaerobic enterotoxemia of saiga is revealed caused by Clostridia pathogen [15].The performed studies showed the isolation of the bacteria Clostridium from the dead saiga.

The gene of ribosomal RNA (rRNA) is considered as highly conserved within the genus and species [16, 17] and may be used as the standard for the classification and identification of Clostridium bacterial isolates. The result of our data revealed that the strains of the bacteria Clostridium, isolated from the saiga in 2010 - 2013 are referred to the Clostridium bacteria of perfringens species.

If an organism is poisoned by toxins produced by Clostridium perfringens, the pathogenesis of the disease is influenced greatly by alpha-toxin, but its exact role in the disease process is not yet fully revealed. All five types

of Clostridium perfringens bacteria (A-E) carry and express the structural gene plc encoding alpha toxin. Alpha-toxin is a secreted zinc metallic enzyme that has both phospholipase C activity, and sphingomyelinase activity [18, 19].

The structure of alpha-toxin is composed of two domains: N - domain (positions 1-250) which contains the catalytically active site and C - domain (positions 251-370) responsible for the binding to membranes [20]. The strains of Clostridium / Saigas / 2010 / ZKO / KZ, Clostridium / Saigas / 2011 / ZKO / KZ, Clostridium / Saigas / 2012 / Kostanay / KZ bacteria Clostridium perfringens, isolated from the saiga in 2010, 2011 and 2012 within the Republic of Kazakhstan showed a very high conservatism according to N and C-domain structures of alpha-toxin. Whereas the N-domain of the alpha-toxin Clostridium / Saigas / 2013 / Kostanay / KZ showed previously unknown 4 amino acid substitutions - Ala13Thr (alanine → threonine), Val47Ile (valine → isoleucine), Ala202Asp (alanine → aspartic acid), Thr205Ala (threonine → alanine), and C-domain shows one amino acid substitution - Ser363Pro (serine → proline). The degree of identity in the N-terminal catalytic domain reaches 98.4% and in C-domain responsible for the binding to membranes the identity makes 99.2%. Perhaps the abovementioned replacements reduce the toxicity of alpha-toxin strain of Clostridium / Saigas / 2013 / Kostanay / KZ. If the death rate of saiga antelopes made 12,000 heads in 2010, 500 heads in 2011, 1000 heads in 2012, it made only 10 heads in 2013. In 2013 the cases of mass death of animals were not observed.

The amino acid substitution at the position of Ile373Val (isoleucine→valine) of Clostridium / Saigas / 2013 / Kostanay / KZ strain is outside the domain. The position 373 of alpha-toxin strains Clostridium / Saigas / 2010 / ZKO / KZ, Clostridium / Saigas / 2011 / ZKO / KZ, Clostridium / Saigas / 2012 / Kostanay / KZ, isolated from the saigain 2010 - 2012 holds isoleucine. However, the substitution of valine for isoleucine in position 373 is conservative one and does not affect the tertiary structure of the protein, as it is located at the area of two coils junction [21]. Thus, the genomic differences in the structure of alpha toxin in Kazakhstan strains Clostridium perfringens in 2010-2012 and 2013. The extractions made 2%.

The strains of Clostridium perfringens, isolated from the saiga in 2010 - 2012 have 99% - 100% genetic relatedness with the strains Clostridium perfringens NRRLB-23782 (DQ184137) and Clostridium perfringens NRRLB-41055 (DQ184174), isolated from bovine. The strains of Clostridium perfringens NRRLB-23782 (DQ184137) and Clostridium perfringens NRRLB-41055 (DQ184174) are isolated during the outbreak of hemorrhagic enteritis bovine. The Kazakhstan strains of 2010 - 2012. The extractions in comparison compared with the strain Clostridium perfringens NRRLB-23782 (DQ184137), isolated from bovine have one amino acid substitution Val43Ala in the structure of alpha toxin.

The strain Clostridium perfringens, isolated from by the saiga in 2013 is closely related to the strain Clostridium perfringens CPA094 (JF298801), isolated from sheep in Iran and Clostridium perfringens A18-Sp38 (DQ838702), isolated from goats in Iran. Thus, it is possible that antelopes were infected by Clostridia disease from external environment, in particular from farm animals.

The catalytic activity of phospholipase C needs zinc ions due to 8 amino acids ofalpha-toxin (Trp1, His11, Asp56, His68, Asp130, His136, His148, and Glu152) [22]. The catalytic activity of all isoforms of phospholipase C depends on calcium ions, for example, the changes in residues that bind calcium ions (Glu32, Asp269, Gly271, Thr272, Asp273, Asp293, Asn294, Gly296, Asn297, Asp298, Asp336, and Ala337) may prevent the formation of membrane complexes [23, 24]. However, the amino acid sequences of alpha-toxin structure in Kazakhstan strains in these positions show the same amino acids. Alape-Girón et al. reported that replacement in Asp269, Asp336, Tyr275, Tyr307 and Tyr331 reduced the toxicity of alpha-toxin [24]. Nagahama et al. reported that the replacement of Asp56, Asp130 and Glu152 resulted in reduction of alpha-toxin toxicity [25]. Such amino acid changes reducing the toxicity in alpha-toxin of the bacterium Clostridium perfringens strains, isolated from the saiga during the period of 2010-2013 were not detected. The part of the alpha-toxin in all four strains of Clostridium / Saigas / 2010 / ZKO / KZ, Clostridium / Saigas / 2011 / ZKO / KZ, Clostridium / Saigas / 2012 / Kostanay / KZ and Clostridium / Saigas / 2013 / Kostanay / KZ makes the bacteria of the genus Clostridium isolated from the saiga in 2010 - 2013 in

the Republic of Kazakhstan. These positions are presented by amino acids Lys56, Ser130, Ala152, His269, Asn275, Gly307, Ser331, Thr336, affecting the important functional properties of alpha-toxin.

CONCLUSIONS

The study revealed genetically variable strains of Clostridium perfringens according to alpha-toxin, isolated from dead saiga. The strains of Clostridium perfringens, isolated in 2010 - 2012 differ from the strain isolated in 2013. The appearance of genetically different strains of Clostridium perfringens is a possible consequence of evolutionary changes in the pathogen genome associated with a long-term persistence in a carrier's body. And also, we cannot exclude the origin of genetically different strains of Clostridium perfringens from external environment, in particular from the farm animals of nearby populated areas, located in saiga habitat.

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