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Fatal outbreak among camels in Kazakhstan associated with *Paeniclostridium sordellii*

An outbreak of a bacterial disease with lethal cases was registered in camels in Kazakhstan in 2018. The disease was characterised by dry, harsh hacking cough and nasal discharge with froathy and bloody liquid. Camels died in a state of hyperexcitement and asphyxia. Investigation of the case has identified the pathogenic form of *Paeniclostridium sordellii* as the main cause of mortality in camels. The purpose of the study is to identify the cause of mortality among camels, bacteriological and molecular genetic characteristics of the pathogen. The methodological basis of the work is collection of biological samples from camels, isolating the pathogen on media, extracting nucleic acids, obtaining libraries for next generation sequencing, bioinformatic analysis of the obtained data, pathogenicity test on mice. This article describes the cultural, genetic and phenotypic characteristics of the isolated Shetpe PS18-01 strain. It was shown that the pathogenesis was mainly characterised by respiratory signs, in contrast to the most common gastrointestinal ones.

Keywords: camel, Kazakhstan, mortality, *Paenilostridium sordellii*, next generation sequencing.

Introduction

Camel breeding plays a significant role in the life of nomadic peoples. Historically, camels in Kazakhstan served as an important source of food, clothing, and transport. The camel livestock size in Kazakhstan is 216,358 heads [1], being an important branch of animal husbandry in the west of Kazakhstan, characterised by semi-desert landscapes. Until now, the health status of the local camel population has been relatively well, but in recent years, due to drought and associated lack of feed and its poor quality, there has been an increase in the morbidity with necrobacteriosis [2] and camelpox [3].

In February 2018, an outbreak of the disease with a high death rate occurred among dromedary camels in the Mangystau region of Kazakhstan. Animals died on the second or third day and mortality among affected herds reached 90 %. Intensive antibiotic therapy has reduced mortality to some extent. Such cases have never been registered before in this region. The primary pathogen *Paeniclostridium sordellii* was identified by applying massive parallel sequencing. The pathogenicity of clostridial infections is based on the release of exotoxins that penetrate the bloodstream, affect the vascular endothelium, mucous and serous membranes, internal organs, cause multiple hemorrhages, destroy the parenchyma of the kidneys, liver, and other organs. Exacerbation of the disease and mortality were recorded primarily in autumn, winter and especially early spring, then enzootic subsides in summer [4].

Experimental

Ethical approval

All research components involving live animals were conducted according to regulations under the legislation “Rules for conducting biomedical experiments, preclinical (non-clinical) and clinical studies (No. 697, 12 November 2007, Republic of Kazakhstan)” and were approved by the SPC of Microbiology and Virology Local Ethics Committee (Approval number: 02-12-109).

A post-mortem examination and sample collection

The post-mortem examinations were carried out on three dead juvenile camels for 3–6 hours. Tissues for further analyses were taken from the liver, lungs, intestines, kidney, spleen, brain and lymph node and delivered to the laboratory.

Cultivation of bacteria in media

The swabs from organs were cultured anaerobically at 38 °C for 18 hr in Kitt-Tarozzi medium (BTN, Russia). The crude cultures were sub-cultured onto blood glucose agar containing 5 % sheep blood and 1 % glucose. After 18–20 hr at 38–40 °C the formation of large colonies with a “fern-leaf” edge and convex dark

centre was observed. The formation of a transparent hemolysis zone was registered. Gas formation was moderate. Colonies with typical growth characteristics were sub-cultured and proceeded with DNA extraction.

Nucleic acid extraction and library preparation for Next Generation Sequencing

Three kinds of libraries were prepared: for sequencing of cultured bacteria, virome analysis, and 16S rRNA analysis. Library preparation for sequencing of bacterial genome started with DNA extraction from bacterial media using the Purelink Microbiome DNA Extraction Kit (Invitrogen, USA) according to the manufacturer's manual. The extracted DNA was fragmented to a size of 400-500 bp using the DNA Fragmentation Kit (New England Biolabs (NEB), USA). Libraries were prepared using the NEBNext Ultra DNA Library Preparation Kit (NEB, USA) according to the manufacturer's protocol.

For virome analysis, pieces of organs were homogenised using Tissuelyser device (Qiagen, Germany) and centrifuged, the supernatants were filtered through a 0.45 um filter (Membrane Solutions, USA) and treated with a mix of nucleases: Benzonase (Sigma-Aldrich, USA), Turbo DNAse, DNAse I, RNAse A, andRNAse T1 (ThermoFisher, Lithuania) and further proceeded with Purelink Viral RNA/DNA Mini Kit (Invitrogen, USA) for nucleic acids extraction. Libraries were prepared using the NEBNext Ultra RNA Library Preparation Kit (NEB, USA) according to the manufacturer's protocol. Library size selection was performed using Ampure XP beads (Beckman Coulter, USA).

16S rRNA libraries were prepared from DNA extracted directly from organs, then proceeded with library preparation using Nextera XT Library Preparation Kit (Illumina, USA) with primers targeting the 16S rRNA gene fragment and containing specific Illumina adapters. The size of all prepared libraries was checked on a Bioanalyzer 2100 instrument (Agilent, Germany). The obtained libraries were subjected to paired-end sequencing on a MiSeqdevice using the reagent kit v3 (Illumina, USA).

Fermentation properties

Carbohydrates fermentation properties were determined using liquid media supplemented with galactose, glucose, arabinose, xylose, lactose, maltose, sucrose, raffinose, mannitol, dulcite, sorbitol, and glycerin. The enzymatic activity was observed for ten days.

Injection of Guinea pigs

The Guinea pigs were intraperitoneally injected with 0.3 ml of diluted bacterial culture. The experimental animals were observed for ten days. The virulence of the isolated cultures was determined by calculating the 50 % lethal dose (LD50). The control group of Guinea pigs was injected with PBS.

Bioinformatic analyses

Quality analysis of obtained NGS output files was performed using FastQC [5] and the reads were bioinformatically analysed using Geneious Prime 2021 software (Biomatters, New Zealand) [6]. Default parameters were used for all software operations unless otherwise specified. Reads from bacterial sequencing libraries were assembled de Novo using Geneious Prime software applying the installed SPAdes 3.15.2 algorithm with default parameters.

Virome sequences were subjected to BLASTn and BLASTx search in the local viral reference database as described in Metavisitor pipeline [7]. Local BLAST hits with lengths ≥100 nucleotides (nt) were considered as significant at E value ≤10e-5, and the potential viral sequences were subjected to aligning with respective viral sequences downloaded from Genbank. The Clustal W algorithm was used for the alignment of obtained sequences in MEGA X software [8].

Reads obtained from 16S rRNA sequencing were analysed on Geneious server (Biomatters, New Zealand), applying the installed algorithm with default parameters.

Results

Clinical signs

Signs of respiratory disease were observed: dry cough, frothy bleeding from the nostrils, and sometimes nosebleeds. Camels were breathing hard, and they stretched their necks to draw in air. Lesions from the gastrointestinal tract in the form of diarrhoea, colic and others were not observed. Faeces were of normal consistency and typical colour. The animals were emaciated, severely depressed.

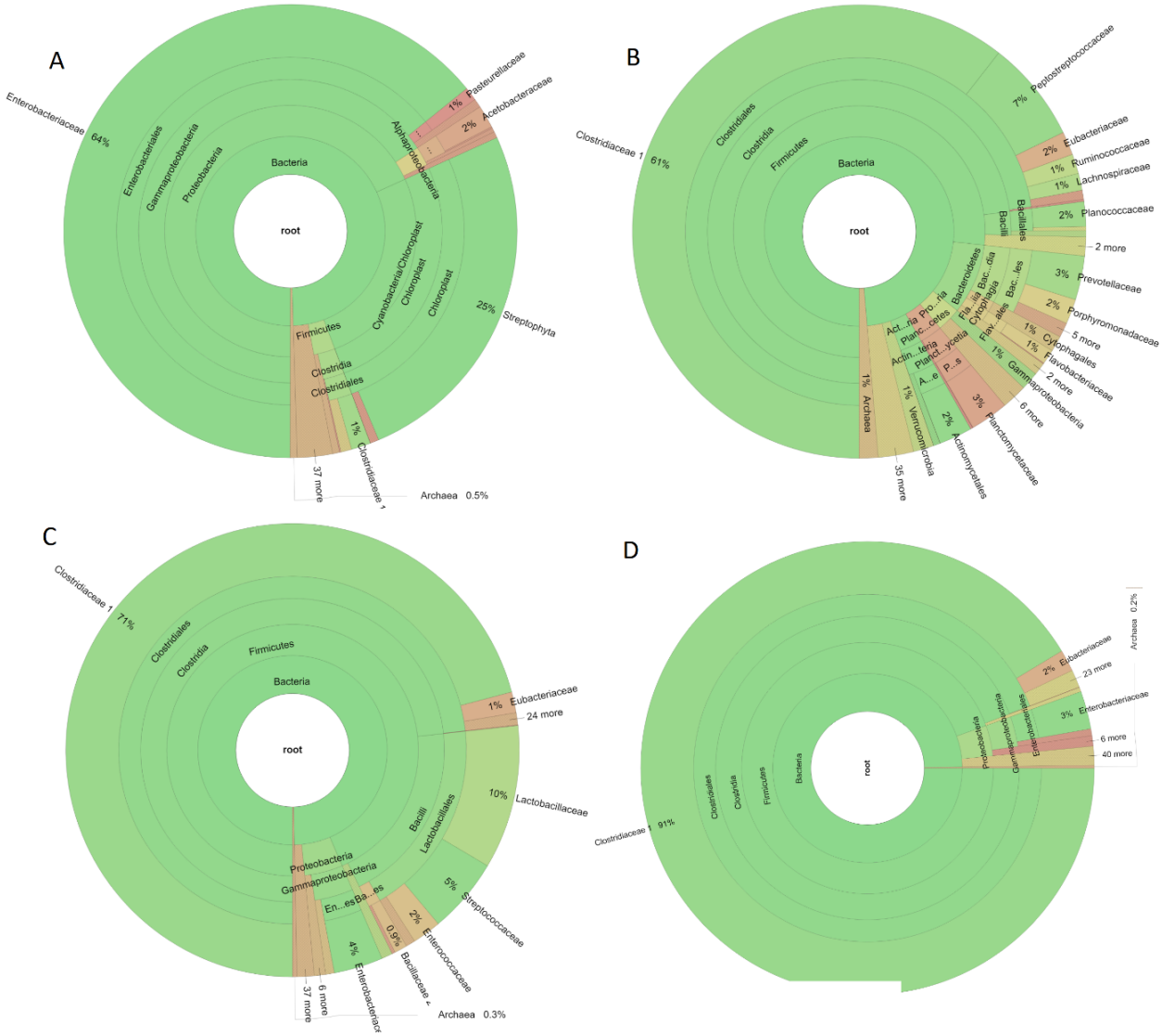
Postmortem examination

Three animals were necropsied, in which a similar pattern of the lesions was observed: lungs of dark brown colour, crepitus was audible when squeezed. At the site of the incision, an abundant foamy liquid mixed with blood flew down. The mucous membrane of the trachea was strongly hyperemic, filled with a frothy liquid. The liver retained sharp edges, flabby consistency, and crumbled when cut. The spleen was dark red, filled with blood, increased in volume.

Palpation of the kidneys revealed a flabby cortical layer, and the kidney capsule was easily released. When cut, the kidney crumbled. The intestines were swollen, the serous membrane was yellowish. In one case, the blood vessels in the intestine were filled with blood. The dura mater of the brain was hyperemic and swollen. The mediastinal lymph node was red and enlarged.

Next Generation Sequencing

The sequence data obtained were trimmed at the 3' and 5' ends with an error probability limit of 0.05. Firstly, the results of 16S rRNA sequencing were analysed (Figure 1). The study of the bacterial diversity in lungs, liver and spleen revealed a clear predominance of 61–91 % of bacteria of the *Clostridiaceae* family, which includes various species of the *Clostridia*genus. Identification of the bacterial species in the intestine showed the predominance of *Enterobacteriaceae* (64 %), a large family of Gram-negative bacteria, most of which are normal members of the gut microbiota, and 25 % constituted Streptophytathat include all land plants.



A. Intestine; B. Lung; C. Liver; D. Spleen

Figure 1. Results of 16S rRNA sequencing of various organs

For the bacterial genome, 1,338,952 sequencing reads were de Novo assembled using Geneious Prime software applying the installed SPAdes3.15.2 algorithm to produce 2,771 contigs. 2,130 of obtained contigs were >500 nucleotidesin size and were taken for further analyses. A BLAST search of the contigs using the

cutoff E value of ≤10e-5 in the non-redundant Genbank database has shown that 92.8 % of them represented *Paeniclostridium sordellii* species (Tab. 1).

Table 1

Characteristics of contigs obtained after de Novo assembling

Organism	Number of Contigs	Percent from all contigs
Paeniclostridiumsordellii	1978	92.8
Entrococcus sp. (E. lactis, E. durans, E. faecalis, E. faecium)	69	3.2
Clostridium perfringens	39	1.8
Clostridium botulinum	19	0.8
TRA asm: Siphoviridae sp.	7	0.3
TRA asm: Myoviridae sp.	5	0.2
Streptococcus sp.	3	0.14
Clostridium chauvoei	2	0.09
Clostridium novyi	2	0.09
Paraclostridiumbifermentans	2	0.09
Clostridium isatidis	1	0.04
Clostridium pasteurianum	1	0.04
Ramboutsia sp.	1	0.04
Sphingorhabdus sp.	1	0.04

The bioinformatics data for virome analysis have shown the absence of any viral reads responsible for the fatal disease (data not shown).

Molecular findings

To differentiate the representatives of the genus *Clostridium* in comparative genomic analyses, three conserved indels in three highly conserved proteins (a four aa insert in DNA gyrase A, a one aa deletion in ATP synthase beta subunit, and a one aa insert in ribosomal protein S2) were identified that are unique to this genus [9]. We comparatively analysed the amino acid sequences of the *Paeniclostridium sordellii* isolate with other representatives of the genus *Clostridium* for three above-mentioned highly conserved proteins (Tab. 2).

Table 2

Comparative genetic characteristics of Clostridium isolates for DNA gyrase A, ATP synthase beta subunit, and ribosomal protein S2 genes

Organism	Gyrase A	ATP Synthase beta subunit	Ribosomal Protein S2
Clostridium perfringens	DMTGDKRHAKALDGIVDI	MAEYFRDQ-GQDV	KELDASNIGAMFVVDPRKEKN
Clostridium botulinum	.....R.....L.S..	.........-....	....CD..............
Clostridium chauvoei	E.....S...EA.I..	.........-....	RN..................
Clostridium beijerinckii	.....A.....S.T..	.......K-....	.......V....I.......
Clostridium kluyveri	...A....I.....P.S..	...H...E-....	.AMN.E.V..L........
Clostridioides difficile	.LVKE.-----IEG.L.L	...H...EQ....	.DMPE.P-..........NI
Shetpe PS18-01	.LVK..K-----..G.S.L	.........E....	.DMPELP-A.I.......NI
Clostridium sordelli	.LVK..K-----..G.S.L	.........E....	.DMPELP-A.I.......NI
Moorella humiferrea	DLVRE.K-----IDG.V.L	.......AE....	.EMRQLP-D.LY......RI

We can see four amino acids deletion in DNA gyrase A protein, an insertion of one amino acid (aa) in ATP synthase beta subunit and deletion of one aa in ribosomal protein S2 homologues in *C. sordellii, C. difficile* and *Moorellahumiferrea* species that differentiate them from the true representatives of the genus *Clostridium* (Clostridium sensustricto). These findings confirm the previous observations about the genetic similarity of *Paeniclostridium sordellii* with *Clostridium defficile* [10].

As mentioned earlier, *C. perfringens* contigs were present in the analysed sample together with *Paeniclostridium sordellii* contigs. Two PCRs with primers to 16S rRNA [11] and the homolog of the alphatoxin of *C. Perfringens* [12] specific only to this species were carried out to rule out the possible presence of *C. perfringens* in the sample. Both PCRs revealed negative results (data not shown).

It is known that the large clostridial cytotoxins (LCC) tcsL and tcs Hgenes of *C. sordellii* are major virulence factors localised on plasmids. Many strains do not contain those genes as they probably lost plasmids

during laboratory subculture [13]. Genome analysis of our *P. sordellii* strain detected only a 475 nucleotide fragment of the plasmid, encoding LCC genes but, possibly, that was a fragment of the phage sequence located on the bacterial genome.

Four other exotoxins were previously suggested as a probable additional virulence factor in this species: a secreted collagenase colA, the cholesterol-dependent cytolysin (CDC) sordelliilysin (sdl), the neuraminidase nanS and phospholipase C [**Ошибка! Закладка не определена.**]. We identified all four genes in our *P. sordellii* strain: sequence of sdl was 97 % identical to sordelliilysine isolated from the closest ATCC9714 strain (Genbank accession No LN679998), 98 % similar to this isolate by colA, 99.8 % similar by nanS and 100 % similar by phospholipase C. Products of these genes are considered as possibly responsible for the lesions observed during the disease.

Fermentation properties

Studying the enzymatic activity of the isolated Shetpe PS18-01 strain has shown the ability to ferment glucose, fructose and maltose. The ability to produce lecithinase differentiates the Kazakh *Paeniclostridium sordellii* strain from similar *C. bifermentans* and *C. difficile* species.

Biological assay on mice for toxicity

The toxicity of the isolated Shetpe PS18-01 strain was assessed by observation of the clinical signs, the intensity of damage to internal organs and the timing of the death of infected animals after inoculation into guinea pigs. The isolate was highly toxic for them. The death of animals was registered 18–20 hours after infection while the control group survived.

Discussion

Clostridia can be found in the soil and the digestive tract of healthy animals without causing any signs of disease. Fatal outbreaks of clostridioses in animals appear to be caused by predisposing factors such as high protein or carbohydrate diets, abrupt change of weather, handling of animals (e.g., transportation, weighing) and physiological condition (fattening). However, the actual reasons that trigger the severe bacterial infestations remain poorly understood [14]. In our investigation of the cause of the outbreak of clostridiosis in camels, no obvious predisposing factors were identified either. We can only assume a possible abrupt change in the diet in the autumn-winter period, which may serve as one of the reasons [15].

Paeniclostridium sordellii was isolated during this outbreak and this strain was recognised as responsible for mass mortality in this region. Major virulence factors tcsL and tcsH genes of *C. Sordellii* located in plasmids were not found during molecular analyses. Possibly they were lost during laboratory subculture. The other four genes colA, sdl, nanS and phospholipase C recognised as potential exotoxins responsible for additional lesions, were detected in our isolate. Biological assay on mice confirmed high toxicity to susceptible animals.

Molecular analyses to compare the Shetpe PS18-01 strain with representatives of other *Clostridium* species were used. Analyses of three conserved genes encoding DNA gyrase A protein, ATP synthase beta subunit and ribosomal protein S2 showed 100 % identity of the Kazakh isolate with the *Paeniclostridium sordellii* species. Together they turned out to be genetically closer to *C. difficile* and *Moorellahumiferrea* species distant from the true representatives of the genus *Clostridium (Clostridium sensustricto)*. Recent classification suggested a new Cluster XI distinct from the current *Clostridiaceae* family involving *P. sordellii, C. difficile, Filifactoralocis, and Peptostreptococcus anaerobius*[**Ошибка! Закладка не определена.**].

Historically, *Paeniclostridium sordellii* has been frequently associated with gas gangrene in humans [16] and gastrointestinal disease in animal species [17]. Literature on *Paeniclostridium sordellii* infection in camels is scarce and only one case of camel clostridiosis associated with this species has been described [18]. *Paeniclostridium sordellii* was often associated with pathology in the gastrointestinal tract of affected animals, but mostly respiratory signs were observed in our case. A study of the microbiome of various organs of camels was carried out to find out the reasons for this phenomenon. 16S rRNA sequencing data showed a very insignificant quantity of *Paeniclostridium sordellii* in the intestine of a camel, while this species was overwhelming in the lungs and other examined organs. This observation is consistent with earlier assumptions that while most *Clostridia* affects the host intestine, C. sordellii primarily causes soft tissue infection [**Ошибка! Закладка не определена.**].

Postmortem examination revealed destruction of the liver and kidney parenchymae, apparently caused by bacterial exotoxins. At the same time, there were no necrotic changes in the intestines, which can be found, for example, in horses affected by *Paeniclostridium sordellii* [19].Now it is difficult to say whether

such a non-gastrointestinal course of the disease is typical only for camels or if this was a single case. Nevertheless, in any case, this fact should be taken into account in diagnostic studies in the future.

The results of this article show the great value of mass parallel sequencing technology, which is useful in such cases with investigating the causes of mass mortality in domestic animals. It is possible to simultaneously identify the genetic characteristics of the pathogen with the determination of its degree of pathogenicity and the involved exotoxins.16S sequencing of different organs allows to obtain information about the approximate localisation of pathogens in the organism and also to determine the composition of the responsible bacteria in case of mixed infections.

Paeniclostridium sordellii poses a sufficient threat to camel farming worldwide and the study of new strains will make a significant contribution to understanding the mechanisms of disease outbreaks.

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Paeniclostridium sordellii-мен байланысты Қазақстандағы түйелер арасындағы өлім

2018 жылы Қазақстанда түйелерде өліммен аяқталатын бактериялық ауру тіркелді. Бұл ауру құрғақ, қатты жөтелмен бірге мұрыннан көбікпен қанаралас сұйықтық бөлінумен сипатталды. Түйелер қатты қозу және тұншығу күйінде өлген. Зерттеудің мақсаты — түйелердің өлу себебін, қоздырғыштың бактериологиялық және молекулалық-генетикалық ерекшеліктерін анықтау. Әдістері: түйелерден биологиялық сынама алу, қоздырғышты қоректік орталарда бөліп алу, нуклеин қышқылдарын шығару, жаңа ұрпақты секвендеу үшін библиотеканы алу, алынған мәліметтерді биоинформатикалық талдау, тышқандарға биосынама жүргізу. Зерттеулер нәтижесінде түйелердің өлімінің негізгі себебі ретінде *Paeniclostridium sordellii* патогенді түрі анықталды. Мақалада бөлінген Шетпе PS18-01 штамының өсу, генетикалық және фенотиптік сипаттамалары берілген. Асқазан-ішек жолдарының жиі кездесетін белгілерінен айырмашылығы, патогенезде тыныс алу белгілері басым екендігі көрсетілген.

Кілт сөздер: түйе, Қазақстан, өлім, *Paenilostridium sordellii*, жаңа ұрпақты секвендеу.

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Вспышка инфекции с летальными исходами среди верблюдов в Казахстане, связанная с *Paeniclostridium sordellii*

Вспышка бактериальной инфекции с летальным исходом зарегистрирована у верблюдов в Казахстане в 2018 году. Заболевание характеризовалось сухим резким отрывистым кашлем и выделениями из носа пенистой и кровянистой жидкости. Верблюды погибли в состоянии гипервозбуждения и удушья. Цель исследования заключалась в выявлении причины смертности среди верблюдов, бактериологической и молекулярно-генетической характеристике возбудителя. Методы: взятие биологических образцов от верблюдов, выделение возбудителя на средах, экстракция нуклеиновых кислот, получение библиотек для секвенирования нового поколения, биоинформационный анализ полученных данных, биопроба на мышах. Результаты: расследование случая выявило патогенную форму *Paeniclostridiumsordellii* как основную причину смертности верблюдов. В данной статье описаны культуральные, генетические и фенотипические характеристики выделенного штамма Шетпе PS 18–01. Показано, что в патогенезе преобладают респираторные признаки, в отличие от наиболее частых желудочно-кишечных.

Ключевые слова: верблюд, Казахстан, смертность, *Paenilostridium sordellii*, секвенирование следующего поколения.

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