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Characteristics of Unique Imported Vibrio cholerae Strains Which Caused Cases of Acute Intestinal Infection in Moscow in 2023

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ABSTRACT

BACKGROUND: In 2023, two cases of acute intestinal infection caused by identical strains of *Vibrio cholerae El Tor* carrying the preCTX prophage and the classical allele of the *tcpA* gene were reported in Russia for the first time. The patients had recently visited Asian countries (Indonesia, Bangladesh, and India). A detailed molecular genetic characterization of the pathogens was required to assess their pathogenic potential.

AIM: To provide phenotypic and genotypic characteristics of *Vibrio cholerae* O1 strains imported to Moscow in 2023 and isolated from patients with acute intestinal infection.

METHODS: In this study, whole-genome sequences (WGSs) were obtained using the *MiSeq Illumina* platform. Bioinformatics analysis was performed using *Vector NTI Advance, BioEdit, BLASTN*, BLASTP, and CARD, as well as the *pygenomeviz* and *biopython* packages.

RESULTS: The studied strains belonged to the biovar *El Tor*, serovar *Ogawa*, and had identical antibiotic resistance profiles. Their genomes contained the preCTX and RS1 prophages with a unique gene composition. RS1 harbored the $rstR^{calc}$ gene (*Calcutta* variant), while the RS2 element of preCTX contained the $rstR^{class}$ gene (classical variant), as well as an additional *orfX* gene of unknown function. Within the complete VPI-1 pathogenicity island, the *tcp* cluster—responsible for the production of toxin-coregulated pili—demonstrated significant differences from prototypes in the *tcpF* and *toxT* genes, although their products retained characteristic active domains. The *tcpA* gene was of the classical type but differed from the prototype by three single nucleotide polymorphisms. The strains also possessed a wide array of intact genetic determinants of pathogenicity factors characteristic for the biovar *El Tor*, sufficient to express its full pathogenic potential.

CONCLUSION: The analyzed *Vibrio cholerae* strains were imported from India, Bangladesh, or Indonesia and are linked by a common source of infection and a transmission pathway. The risk of further importation of such or similar strains persists, and the characterized isolates can be used as reference strains for cholera surveillance studies in the Russian Federation.

Keywords: *Vibrio cholerae* O1; preCTX; RS1; TCP; pathogenicity factors; bioinformatics analysis; pathogenetic potential.

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Характеристика уникальных завозных штаммов холерных вибрионов, вызвавших в 2023 году в Москве случаи острой кишечной инфекции

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АННОТАЦИЯ

Обоснование. В 2023 году в России впервые были зарегистрированы два случая острой кишечной инфекции, вызванные идентичными штаммами *Vibrio cholerae* Эль-Тор, содержащими профаг preCTX и классический аллель гена *tcpA*. Заболевшие накануне посетили страны Азии (Индонезию, Бангладеш, Индию). Для определения патогенетического потенциала возбудителей требовалась их детальная молекулярно-генетическая характеристика.

Цель исследования — фенотипическая и генотипическая характеристика штаммов *Vibrio cholerae* O1, завезённых в Москву в 2023 году и выделенных от больных острой кишечной инфекцией.

Материалы и методы. В работе использованы полногеномные сиквенсы (WGSs), полученные на платформе MiSeq Illumina. Биоинформационный анализ выполняли с помощью программ Vector NTI Advance, BioEdit, BLASTN, BLASTP, CARD, пакетов pygenomeviz, biopython.

Результаты. Исследуемые штаммы относились к биовару Эль-Тор, серовару Огава, имели одинаковые спектры антибиотикорезистентности. В их геномах присутствовали профаги preCTX и RS1 с уникальным составом генов. RS1 содержал ген $rstR^{calc}$ (Калькутта), а RS2-элемент preCTX — $rstR^{class}$ (классический), а также дополнительный ген *orfX* с неизвестной функцией. В полном острове патогенности VPI-1 *tcp*-кластер, ответственный за продукцию токсин-корегулируемых пилей, имел существенные отличия от прототипов по генам *tcpF* и *toxT*, но их продукты сохранили характерные активные домены. Ген *tcpA* относился к классическому типу, но отличался от прототипа тремя однонуклеотидными полиморфизмами. У штаммов также обнаружен обширный набор характерных для вибрионов Эль-Тор интактных генетических детерминант факторов патогенности, достаточных для реализации патогенетического потенциала.

Заключение. Изученные штаммы холерных вибрионов были завезены из Индии, Бангладеш либо Индонезии и связаны единым источником заражения и фактором передачи инфекции. Риск завоза таких и подобных штаммов сохраняется, и охарактеризованные изоляты могут быть использованы в качестве эталонных при мониторинговых исследованиях на холеру на территории Российской Федерации.

Ключевые слова: *Vibrio cholerae* O1; preCTX; RS1; TCP; факторы патогенности; биоинформационный анализ; патогенетический потенциал.

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BACKGROUND

Over the past 15 years, rare cases of cholera caused by strains of epidemic significance have been sporadic in Russia and always related to importations from India. Their causative agents belonged to the Haitian (2010) and post-Haitian (2012, 2014, 2023) groups, which hold a dominant position in the global etiology of cholera [1, 2]. In 2023, Moscow recorded its first two cases of acute intestinal infections caused by non-toxigenic (lacking cholera toxin genes *ctxAB*) V. cholerae strains, which carried the preCTX prophage and the classical-type tcpA gene encoding the structural subunit of toxin-coregulated pili. The *tcpA* gene differed from the prototype by three single nucleotide polymorphisms (SNPs). The patients, as part of a group of Russian citizens, visited Indonesia, Bangladesh, and India during a business trip, returning to Moscow on a special flight from Delhi shortly before admission. Notably, a member of this group, a Russian citizen, developed cholera due to a toxigenic post-Haitian strain, suggesting an independent source of infection. The second toxigenic strain was isolated in the same year in the Tambov Region as a result of independent importation by Indian citizens.

A phylogenetic analysis of whole genome sequences (WGSs) showed that both of the toxigenic isolates were grouped on the dendrogram with a number of strains isolated in 2021–2023 in foreign countries (Africa, Australia, Pakistan, US), whereas the non-toxigenic strains carrying the preCTX prophage formed a separate cluster, which was distanced from all the others, i.e. they had a unique genotype [2]. These strains were etiological agents of acute intestinal infections, so the initially published brief data clearly required more detailed characterization. This was needed to perform a bioinformatic analysis of individual virulence factor genes and their genomic arrangements (clusters and genomic islands) to assess their potential for expression, which is a critical determinant of virulence potential and etiological hazards.

AIM

The work aimed to provide phenotypic and genotypic characteristics of *Vibrio cholerae* O1 strains imported to Moscow in 2023 and isolated from patients with acute intestinal infections.

MATERIALS AND METHODS

We studied strains of *V. cholerae* O1 isolated in 2023 in Moscow from hospitalized patients with acute intestinal infections who had returned from a business trip to Southeast Asian countries. Strain 132 was obtained from one patient, and three almost identical subcultures of another strain (226, 228, and 264) were obtained from another patient. The strains were stored lyophilized in the collection of live cultures of the Reference Center for Cholera Monitoring at the Rostov-on-Don Anti-Plague Institute. After subculturing from vials, the cultures were tested for all previously characterized properties. This assessment included phenotypic and genotypic markers, including antibiotic susceptibility/resistance, with PCR testing performed according to Guidelines MUK 4.2.3745-22[1].

We used WGSs of these strains obtained using the MiSeq Illumina platform (Illumina Inc., US). We identified genetic determinants of pathogenicity and persistence in WGSs using the BLASTN 2.2.29 (http://blast.ncbi.nlm.nih.gov) and BioEdit 7.2.5 (http://www.mbio.ncsu.edu/bioedit) software. Antibiotic resistance was determined using the CARD database (https://card.mcmaster.ca) and a set of relevant genes found in cholera vibrios described earlier [3]. Genes and their translation products were analyzed using components of the Vector NTI Advance 11 software package (Invitrogen). We searched for homologous amino acid sequences of proteins and identified potential active domains using the BLASTP program (http://blast.ncbi.nlm.nih.gov). The nucleotide sequences of *V. cholerae* strains N16061 of the El Tor biovar (AE003852, AE003853) and O395 of the classical biovar (CP045718, CP045719) were used as prototypes. Manipulations with cluster sequences and data visualization were performed using Python scripts and the pygenomeviz and biopython packages (https://github.com/moshi4/pyGenomeViz).

Ethics Approval

Not applicable.

RESULTS

The strains under study had all the phenotypic characteristics of the *V. cholerae* species and belonged to the El Tor biovar and Ogawa serovar. All of them had the same antibiotic sensitivity/resistance profile: they were sensitive to ciprofloxacin, gentamicin, ceftriaxone, chloramphenicol, doxycycline, tetracycline, clarithromycin, azithromycin, cefotaxime, moxifloxacin, and cefaclor and resistant to ampicillin, rifampicin, tobramycin, streptomycin, amikacin, and nalidixic acid.

Previously, all genes of the preCTX prophage, the RS2 element (*rstRAB*) and additional pathogenicity factors such as *cep* (core encoded pilin), *ace* (accessory cholera enterotoxin), and *zot* (zonula occludens toxin), were identified in WGSs of the study strains, and the C-terminal sequence of the latter differed significantly from that of the prototype gene included in the CTX prophage in the genomes of toxigenic strains. This is typical for preCTX, which was considered to be a precursor of CTX, which, together with the *ctxAB* cholera toxin genes, acquired a new (canonical) *zot* end [4]. The prophage also contained the *psh* and *orfU* ($pIII^{CTX}$) genes; however, these determinants were located across several short contigs with

[1] Guidelines MUK 4.2.3745-22. 4.2. Control methods. Biological and microbiological factors. Methods for laboratory diagnostics of cholera. Available at: https://docs.cntd.ru/document/350413501. Accessed on: November 15, 2024.

overlapping terminal sequences. In this study, we assembled the complete sequence of preCTX and RS1 prophage (Fig. 1).

In its *RS2* element, the preCTX prophage had the classical type rstR gene and an additional gene, which we designated as *orfX*. Most Vibrio cholerae isolates lack this gene; however, its homologs have been identified in specific strains from the US National Center for Biotechnology Information (NCBI) gene database, in particular in isolates from Taiwan (2002) and Uganda (2015). Their translation products (HAS3381368, HAS3410378, PYC77001) did not have potential active domains but were annotated as a "chemotaxis protein" with an unspecified function.

Interestingly, the 180-bp *rstR* gene of the RS1 prophage belonged to the Calcutta type, which is typically associated with O139 serogroup vibrios [5, 6] but has also been occasionally reported in *V. cholerae* O1 strains (WP_000201563, EGQ7979904, EGQ8141952). In the study strains, this prophage was located on the small chromosome, whereas hybrid genome assembly is required to determine the localization of preCTX.

Both pathogens have a complete Vibrio Pathogenicity Island 1 (VPI-1), which has *AldA* clusters with *aldA*, *tagA* genes and open reading frames with an unspecified function; *tcp*, which is responsible for the production of adhesion pili; and *Acf*, which includes genes for additional colonization factors [7]. The core *tcp* cluster showed overall similarity to those of El Tor and classical one; however, its *tcpF* gene diverged so dramatically from prototypes that it was undetectable by BLAST search and was only identified in WGSs. Despite having a different amino acid profile, the product of its translation had the same active domain, and its homologs, 99% identical, were found in the NCBI database (EGR4403235, NOE84825, NOE95797, NOF00903, NOF18360). The *toxT* gene showed significant divergence from the prototypes, particularly in its proximal region (1–436 bp). The distal region (437–831 bp) contained 17 SNPs, most of which were silent mutations. The C-terminal domain (144–277) contained only two substitutions, whereas the N-terminal domain (1–143) had numerous changes. However, the protein retained all its active domains and had full homologs in the NCBI database (6P7R_A, AAG31795, EHE0024350, EGR4117644, EGR1124498, EGR4402848), which was not surprising because its active domains were concentrated mainly at its distal end.

Fig. 2 shows products of the *tcp*-cluster genes of the study strains compared with those of the typical strains of the classical biovar and El Tor. Differences in the structure of these and some other proteins can be seen.

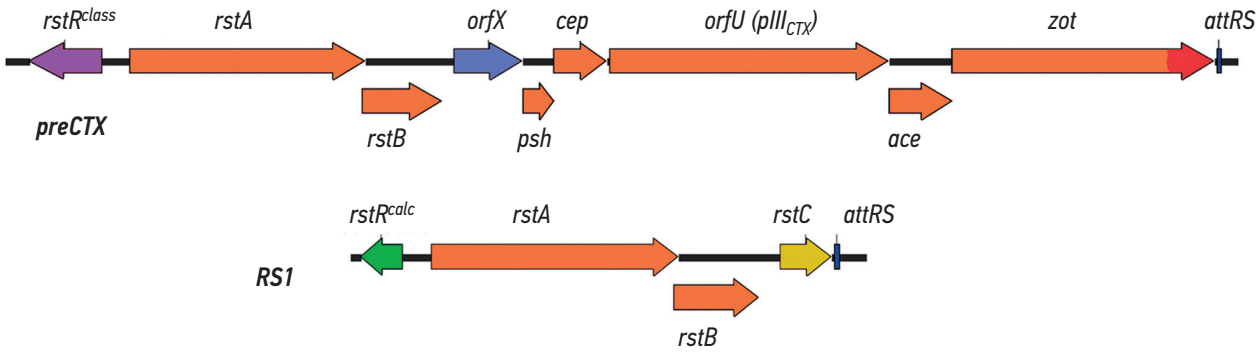

Fig. 1. Structure of preCTX and RS1 prophages of imported strains isolated from patients in Moscow (2023).

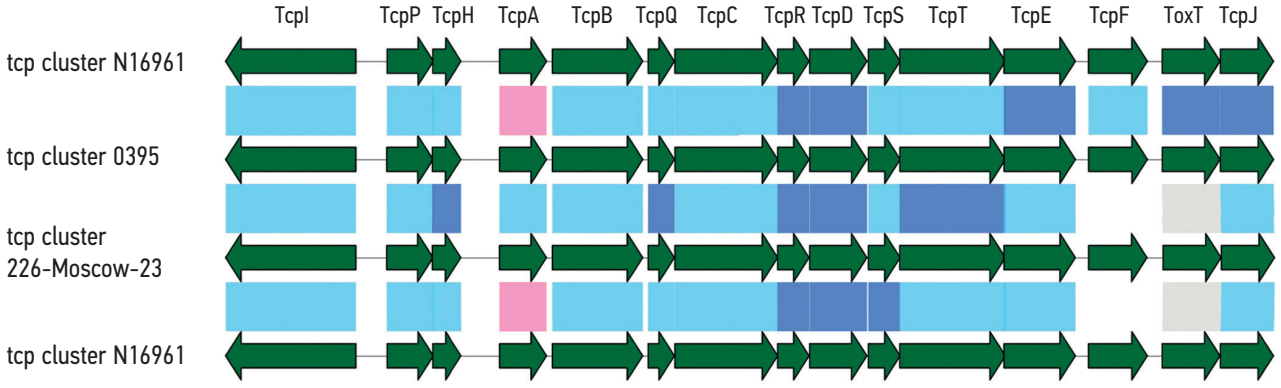

Fig. 2. Comparison of amino acid sequences of *tcp* cluster gene products from *V. cholerae* strains N16961, 0395, and 226 (Moscow, 2023). Connecting lines in blue, light blue, pink and gray indicate 100% identity, and identity in the range of 95.0%–99.9%, 80.0%–84.9%, and 70.0%–79.0%, respectively. The coverage parameter for pairwise comparison of proteins (70%) was applied. We did not perform comparison for sequences with coverage of less than 70%; no connective lines are shown.

As noted above, the *tcpA* gene belonged to the classical type but differed from the prototype by the presence of 3 SNPs (G117T, A530C, A625G), two of which were missense mutations that led to substitutions in the protein (i.e. N177T and K209E). Its homologs were found in the NCBI database, differing only by one (i.e. the first) substitution (i.e. ACK75631, AHY02152, EJL6303812, KEH05472, KNH49515). These homologs belonged to strains of the non-O1/non-O139 serogroups, including the toxigenic V52, which was the causative agent of a large epidemic outbreak in Sudan in 1968.

The VPI-2 pathogenicity island was present in its complete form and showed near-identity to the prototype, except for a few SNPs. Its *nan-nag* region demonstrated 100% homology to the reference sequence.

The RTX cluster responsible for synthesizing a high-molecular-weight self-processing actin modulator called the MARTX cytotoxin was 99% homologous to that of the El Tor reference strain. The *rtxA* toxin gene had the same length as the El Tor prototype and contained 13 SNPs. None of these SNPs resulted in a premature stop codon; six were silent (synonymous); the remaining seven were missense mutations leading to non-synonymous amino acid substitutions in the protein. These substitutions did not disrupt the protein structure or its active domains.

There was no gene cluster for the Type 3 Secretion System (T3SS); however, T6SS clusters were present, namely the main cluster and auxiliary clusters (AUX1, AUX2, and AUX3). They contained determinants for structural components and effectors of this secretion system and were 99%–100% homologous to their reference prototypes.

The *msh* cluster, which is responsible for producing mannose-sensitive pili (Mannose-Sensitive Hemagglutinin, MSHA), was 99% identical to the prototype. The clusters responsible for synthesizing polysaccharides (Vibrio Polysaccharide, VPS) and the *vps-I-rbm-vps-II* proteins showed more differences and matched only 97.5%.

The genes encoding additional pathogenicity factors, including hemolysin/cytolysin HlyA, cytotoxic factor CefE1, hemagglutinin/protease HA/P, collagenase VchC, metalloprotease PrtV, serine proteases VesA, VesB, VecC, IvaP, and RssP; outer membrane proteins OmpU, OmpT, and OmpW; and transcriptional regulators ToxR, HapR, LuxO, CdgA, AphA, AphB, Hns, and CytR, were either identical or highly similar to their prototypes.

There were no pathogenicity islands VSP-I and VSP-II; only their flanking genes (VC0141 and VC0153 for VSP-I; VC0459 and VC0517 for VSP-II, respectively) were present. At the VSP-II insertion site, a sequence corresponding to *tRNA-Met8* was identified.

No genes for the *chxA* cholix toxin and the *stn/sto* heat-stable toxin were identified.

As for drug resistance determinants, the CARD program detected only the *alm* operon (resistance for polymyxin), the β-lactamase gene *varG* (for carbapenems), the *qnrVC4* gene (for fluoroquinolones), as well as CRP efflux pumps. Using the BioEdit software, we found determinants of *VcmA*, *B*, *D*, *H*, *N*, *VcrM*, *VC1634* efflux pumps.

DISCUSSION

A comprehensive genomic analysis of imported clinical strains confirmed previous findings regarding their uniqueness, demonstrating no genetic relatedness to other isolates and the formation of a distinct, phylogenetically distant cluster [2]. The uniqueness concerned primarily the structure of the preCTX and RS1 prophages, because the NCBI database did not contain any identical complete sequences for them, although some matches were found for individual genes. This is not surprising, because this reflects the extreme heterogeneity of these prophages and their mosaic structure. Different types of preCTX prophages are known to exist not only as individual copies but also to form tandems within a single genome, integrating into both the large and small chromosomes. Tandems may include preCTX-preCTX, preCTX-CTX, CTX-RS1-preCTX, and other variants, whereas prophages may be represented by both identical and different types [8, 9]. Prophage types are classified depending on the structure of the RS2 element genes, primarily the *rstR* gene, which has the largest number of alleles. The most famous of them include $rstR^{ET}$ (El Tor), $rstR^{class}$ (classical), $rstR^{calc}$ (Calcutta), $rstR^{env}$ (environmental) [10, 11], but in recent years many additional alleles have been described [6, 9]. The coexistence of prophages with different alleles likely results not from simultaneous infection by two distinct CTXφ/preCTXφ phages [12], but rather because each prophage provides immunity only against superinfection by homologous phages but not by other phage types [5, 6].

As noted above, the $rstR^{class}$ gene was detected in the RS2 element of the study strains, and $rstR^{calc}$ was detected in the RS1 prophage. The origin of the "extra" *orfX* gene, which is localized downstream of *rstB* in preCTX of the study strains, remains unknown. Only a single case of an additional gene, designated *rstU*, in the RS1 prophage has been reported in the published data. This strain was isolated from the environment in China (KP768424) [8]. It was completely homologous to the gene encoding fumarate reductase subunit D and had nothing in common with the *orfX* we found, the product of which was described as a "chemotaxis protein" in the NCBI database.

Another distinctive feature of the study strains was an unusual architecture of their $tcpA^{class}$ gene, which diverged from the prototype by three SNPs, and significant alterations in *tcpF* and *toxT* genes of the *tcp* cluster, though their translated products still had all functional domains. In the NCBI database, we found five 99% homologs for the first variant and five 99% homologs for the second one; the third variant had six 100% matches. Only one strain contained both the second and third variants simultaneously. All others represented distinct strains, none of which carried the aforementioned TcpA variant. This is quite consistent with the mosaic structure of the *tcp* cluster, where *tcpA* has the greatest variability [7, 13].

Different authors have described multiple alleles of this gene and some others in this cluster [14, 15]. It has been repeatedly suggested that genetic variability within both VPI-1 and CTX/preCTX elements plays a crucial role in V. cholerae evolution by fostering new clonal variants with enhanced persistence, pathogenicity, and epidemic potential through interstrain genetic exchange [14–16].

Acute intestinal infections caused by *V. cholerae* O1 strains harboring the preCTX prophage were identified in Russia for the first time. Previously, they were occasionally found only in environmental waters (Moscow, 1977; Crimea, 1991; Rostov-on-Don, 1982, 1987, 2007) or isolated from patients in Uzbekistan (1988, 1990). The only clinical isolate (Rostov-on-Don, 1981) belonged to the nonO1/nonO139 (O8) serogroup [17]. Detected sporadically in Russia, preCTX$^+$VPI$^+$ strains represent imported cases originating from other countries outside Russia, likely Asian, where they are more widely distributed and cause human infections. Specifically, such strains have been detected in China among isolates belonging to genetic lineages L3b and L9. In their characterization, the authors designated these strains as epidemic with potential for global spread [18, 19]. Indeed, two preCTX$^+$VPI$^+$ strains of the L3b lineage were isolated from patients in Switzerland [20]. Regardless of their isolation source and serogroup, all previously studied strains of our collection with this genotype caused a significant enteropathogenic effect in the model of infant rabbits, which sometimes resembled the cholera effect [17]. Their increased pathogenetic potential is directly related to the presence of preCTX, which contains genes of the Zot and Ace toxins and additional colonization Cep factor, and to the VPI-1 island, which includes determinants of not only the key colonization factor TCP but also additional Acf. Furthermore, they usually have a virtually complete "standard" set of intact determinants of pathogenicity typical for El Tor vibrios such as VPI-2 island, T6SS, RTX, *msh* clusters, genes for hemolysin *hlyA*, cytotoxic toxin *cef*, *hapA*, *prtV*, *vchC* metalloproteases, *vesA*, *vesB*, *vecC*, *ivaP*, *rssP* serine proteases, *ompU*, *ompT*, *ompW* outer membrane proteins; each of them can contribute to pathogenesis [3].

As for drug resistance determinants, very few were found in WGSs, which did not reflect phenotypic resistance *in vitro*. Resistance to ampicillin could be associated with the *varG* β-lactamase gene, whereas *qnrVC4* did not result in resistance to fluoroquinolones (ciprofloxacin, moxifloxacin). No genes associated with resistance to rifampicin, tobramycin, streptomycin, or amikacin were found. These strains likely possess other, as-yet-undescribed resistance determinants, or their resistance may be mediated by efflux pumps [21]. Such inconsistencies between phenotype and genotype were also found for other strains [3, 22, 23]. This is why, when selecting the most effective etiotropic therapy for acute intestinal infections, health professionals should focus mainly on the phenotype of the isolate.

The pathogens were likely to originate in India, though infection could potentially have occurred in either Indonesia or Bangladesh, where the patients shared the same hotels and traveled on the same special flight, suggesting possible transmission through foodborne exposure, person-to-person contact/fomites, or waterborne routes (including iced beverages). Reports on Vibrio cholerae detection (including toxigenic and multidrug-resistant strains) in food-grade ice and iced beverages [24, 25], beverage production line equipment [26], and street-vended fruits and vegetables [27] have originated predominantly from Indonesia. Meanwhile, cholera vibrios have also been detected in street-vended foods in Bangladesh, although their characteristics were not reported;[2] in vegetables and greens, only unspecified *Vibrio spp.* were identified without species-level determination [28]. In India, similar studies of salad vegetables found many pathogenic bacteria, but vibrios were not mentioned [29]. However, these studies were limited and irregular, whereas the risk of foodborne or waterborne cholera transmission is unequivocal. For example, an outbreak of cholera in Vietnam in 2010 was associated with consuming iced tea [30].

CONCLUSION

The bioinformatic analysis of whole-genome sequences from *V. cholerae* O1 strains isolated in 2023 from two patients with acute intestinal infections, who had returned to Moscow after a short-term visit to Indonesia, Bangladesh, and India, revealed the composition and structural features of their genetic determinants, including gene clusters, genomic islands, and prophages. The genetic identity of the pathogens suggested a single source of infection and a common transmission factor. We called these strains unique strictly in the sense that their genomic elements, including the preCTX and RS1 prophages and *tcp* cluster, lacked complete homologs among both our collection strains and those available in the NCBI database, despite some partial matches being found. The uniqueness lies rather in the specific allele combinations within these genetic elements. From this perspective, many other isolates with different combinations could also be considered unique; however, these particular variants represent the first such strains ever imported into Russia. With their extensive repertoire of intact pathogenicity determinants enabling full pathogenic expression, these strains constitute a potential threat to public health.

The risk of further importation of such or similar strains persists, and the characterized isolates can be used as reference strains for cholera surveillance studies in Russia.

ADDITIONAL INFORMATION

Authors' contribution. E.V. Monakhova—analysis of literature and whole-genome sequencing data, writing the text and preparing the article for publication; V.D. Kruglikov—data analysis, discussion of results, editing the

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article; O.A. Podoinitsyna—comparative analysis of protein structure, preparation of figures; A.S. Vodopyanov—whole-genome sequencing, identification of genetic determinants; N.B. Nepomnyashchaya—phenotypic and PCR identification of the studied strains; A.V. Evteev—determination of antibiotic resistance; N.E. Gaevskaya—collecting and analysis of information on the importation and isolation of strains, their characteristics. Thereby, all authors provided approval of the version to be published and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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