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DETECTION AND PHYLOGENETIC ANALYSIS OF *CLOSTRIDIUM VENTRICULI* IN AUTISTIC CHILDREN

Hasan A. H. [a],

Lazim H. H. [a]

[a] Ibn Sina University of Medical and Pharmaceutical Sciences, Baghdad, Iraq.

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ВЫЯВЛЕНИЕ И ФИЛОГЕНЕТИЧЕСКИЙ АНАЛИЗ Clostridium VENTRICULI У ДЕТЕЙ С АУТИЗМОМ

Хасан А. Х. [1],
Лазим Х. Х. [1]

[1] Университет медицинских и фармацевтических наук Ибн Сины, Багдад, Ирак.

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Abstract

Background: Autism spectrum disorder (ASD) is characterized by repetitive behaviors. There is evidence that gut flora imbalance may cause GI difficulties in autistic people. Gastrointestinal (GI) issues are associated with *Clostridium ventriculi (C. ventriculi*).

Aim: The purpose of this study was to use 16S rRNA gene sequencing to identify and genetically describe *Clostridium ventriculi* in fecal samples from children with autism.

Materials and Methods: A case-control study was done on fecal samples collected from 50 children diagnosed with autism. Also, samples were taken from 50 children who were not autistic as a control group. Using the FavorPrep Genomic DNA Mini Kit, DNA was extracted. PCR was used to amplify the 16S rRNA gene using the universal primers 27F and 1492R. After the PCR products were sequenced, BLAST and BioEdit tools were used to check the sequences for homology. The MEGA program was used for phylogenetic analysis.

Results: Based on PCR results, 10% (5/50) of the 50 samples of autistic children that were examined proved positive for *C. ventriculi*, and all control group were negative for this bacteria. Genetic polymorphisms were indicated by specific nucleotide transitions and transversions that were discovered by sequencing. The Iraqi isolates and global samples exhibited a high level of genetic similarity (99%) according to phylogenetic analysis, indicating a recent common ancestor and potential clonal expansion.

Conclusions: The discovery of *C. ventriculi* in autistic children raises the possibility of a connection between this bacteria and gastrointestinal problems linked to ASD.

Keywords: *Clostridium ventriculi*, autism spectrum disorder, phylogenetic analysis, 16S rRNA gene.

10.15789/2220-7619-DAP-17875

Введение: Расстройство аутистического спектра (РАС) характеризуется повторяющимся поведением. Существуют доказательства того, что дисбаланс кишечной флоры может вызывать проблемы с желудочно-кишечным трактом (ЖКТ) у людей с аутизмом. Проблемы с ЖКТ связаны с Clostridium ventriculi (C. ventriculi).

Цель: Целью данного исследования было использование секвенирования гена 16S рРНК для идентификации и генетического описания Clostridium ventriculi в образцах кала детей с аутизмом.

Материалы и методы: Исследование случай-контроль было проведено на образцах кала, собранных от 50 детей с диагнозом аутизм. Кроме того, образцы были взяты у 50 детей, не страдающих аутизмом (контрольная группа). Образцы ДНК выделяли с применением набора FavorPrep Genomic DNA Mini Kit. ПЦР использовалась для амплификации гена 16S рРНК с использованием универсальных праймеров 27F и 1492R. После секвенирования продуктов ПЦР были использованы базы данных BLAST и BioEdit для проверки последовательностей на гомологию. Программа MEGA использовалась для филогенетического анализа.

Результаты: на основании результатов ПЦР 10% (5/50) из 50 обследованных образцов детей-аутистов оказались положительными на C. ventriculi, а все образцы контрольной группы были отрицательными. Генетические полиморфизмы были выявлены с помощью специфических нуклеотидных переходов и трансверсий, обнаруженных при секвенировании. Иракские изоляты и зарубежные образцы показали высокий уровень генетического сходства (99%) согласно филогенетическому анализу, что указывает на недавнего общего предка и потенциальное клональную экспансию.

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Выводы: обнаружение C. ventriculi у детей-аутистов повышает вероятность связи между этой бактерией и желудочно-кишечными нарушениями, связанными с РАС.

Ключевые слова: Clostridium ventriculi, расстройство аутистического спектра, филогенетический анализ, ген 16S рРНК.

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1 Introduction

Repetitive habits, limited interests, difficulties interacting with others, and communication difficulties are all hallmarks of autism spectrum disorder (ASD). It is a condition with genetic origins [4].

The community of around 10^{14} bacteria makes up the gastrointestinal (GI) microbiota. There are 100 times as many genes in these bacteria's genetic material, or microbiome, as there are in the human genome. More than 10^3 species can be found in the human gut ecology. The GI microbiota influences the onset of disease and contributes to health maintenance [5].

This microbial community helps break down components of our diet in the gut and offers protection against viruses, supports immune system training, and impacts gastrointestinal maturation. Additionally, microbes have a major role in the emergence of many diseases [3, 36].

The composition of the gastrointestinal (GI) microbiota is primarily ascertained by age, genetic factors, and nutrition [27].

Several individuals with autism display various gastrointestinal disorders, such as diarrhea, constipation, gas retention, abdominal pain, and discomfort. It is possible that aberrant gut microflora contributes to these issues. Multiple publications have shown an association between *Clostridium* and these difficulties [10].

Clostridium ventriculi (C. ventriculi), alternatively referred to as *Sarcina ventriculi* (*S. ventriculi*), is a rare, anaerobic, non-motile, Gram-positive coccus that ferments carbohydrates. It thrives and proliferates in acidic environments [31].

C. ventriculi, a member of the family Clostridiaceae, is derived from the Latin word "sarcina," which means "package," due to its typical formation of tetrads or octets. The precise pathogenic role of *C. ventriculi* in humans remains uncertain [22].

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10.15789/2220-7619-DAP-17875

This bacterium is linked to serious gastrointestinal issues, especially in people who have obstruction of the gastric outlet or delayed stomach emptying. It is associated with acute blood loss anemia, stomach perforation, emphysematous gastritis, and, infrequently, gastric or colorectal cancer in adults [6, 8, 11].

Although less frequent, pediatric cases exhibit comparable symptoms, with documented occurrences of emphysematous gastritis–related fatal stomach rupture, frequently preceded by persistent vomiting and gastric distension [29, 22].

The bacterium's capacity to digest carbohydrates and produce gas that worsens tissue necrosis and ischemia puts both groups at risk for potentially fatal consequences [29, 14].

Histopathological identification of its characteristic cuboid tetrads in biopsies is necessary for diagnosis [29, 22].

The usual course of treatment includes acid suppression, management of underlying motility issues, and antibiotics (such as metronidazole and penicillins) [6, 9, 14].

Through toxin-mediated pathways and dysbiosis of the gut microbiota, *Clostridium* species are linked to the onset and severity of autism spectrum disorder (ASD). According to research, children with ASD have higher levels of Clostridium (e.g., *Clostridium perfringens*, *Clostridium bolteae*, and *Clostridium histolyticum*) than controls do. This is especially true for strains that produce β2-toxin and neurotoxic metabolites like 3-(3-hydroxyphenyl)-3-hydroxypropionic acid (HPHPA), which interfere with dopamine metabolism and deplete catecholamines in the brain [3, 15, 17].

By cleaving proteins like synaptobrevin, clostridial toxins, such as *C. tetani*'s tetanus neurotoxin (TeNT), can travel through the vagus nerve and enter the central nervous

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system, affecting synaptic function and resulting in behavioral and neurodevelopmental abnormalities [18].

Furthermore, *Clostridium* species generate phenolic compounds and short-chain fatty acids (like propionic acid) that worsen intestinal permeability, neuroinflammation, and oxidative stress, which promotes the systemic spread of neuroactive metabolites [3, 15, 18].

The gut-brain axis may be further disrupted by environmental variables, such as glyphosate exposure, which may selectively suppress good gut bacteria and encourage the growth of *Clostridium* [3].

Vancomycin and fecal transplants, two interventions that target the gut microbiota, temporarily lower *Clostridium* levels and alleviate symptoms of ASD, highlighting their pathogenic involvement [17, 18].

In children with autism spectrum disorder (ASD), gastrointestinal issues have been closely linked to *Clostridium* species, especially *Clostridium histolyticum* (clusters I and II), *Clostridium bolteae*, *Clostridium perfringens*, *Clostridium paraputrificum*, and *Clostridium difficile*. Research has repeatedly demonstrated that ASD patients' stools contain higher concentrations of these species than those of neurotypical controls. For instance, children with gastrointestinal symptoms who had ASD were far more likely to have *C. perfringens* strains that produced β2-toxin genes, which may be a contributing factor to the gut problems associated with ASD. Furthermore, whereas neurotypical youngsters had distinct *Clostridium* profiles, ASD patients were the only ones to have C. bolteae and C. paraputrificum [3, 19].

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10.15789/2220-7619-DAP-17875

Molecular approaches in microbial ecology allow for the examination of intestinal flora composition without the need for culturing. This is accomplished using particular primers, yielding extremely sensitive, practical, and quick results [26].

The purpose of this study was to use 16S rRNA gene sequencing to identify and genetically describe Clostridium ventriculi in fecal samples from children with autism.

Materials and Methods

Specimens

The sample for this case-control study included 50 patients with ASD diagnoses, 40 of whom were male and 10 of whom were female, ages 2 to 8. They were chosen from two nearby facilities that specialize in treating autism. Samples were also taken from 50 children who were not autistic as a control group.

The study excluded children with serious head traumas, neurological disorders, severe physical abnormalities, and gastrointestinal problems like bloating, gas, indigestion, constipation, or recurrent diarrhea. Additionally, the children had not been administered antibiotics or functional foods (probiotics, prebiotics) for at least a month prior to the sampling.

Sampling

The fecal sample was taken before breakfast after each child fasted overnight. Three successive fecal samples were collected from each child. The samples were placed in containers made of sterile plastic, then suspended and stored at a temperature of -70°C for subsequent DNA extraction.

DNA Extraction

Following the manufacturer's recommendations, genomic DNA was extracted from fecal samples using the QIAamp® DNA Stool Mini Kit (Qiagen, Germany). Electrophoresis on a 1% agarose gel was used to verify the existence and quality of the isolated DNA.

ISSN 2220-7619 (Print)
ISSN 2313-7398 (Online)

10.15789/2220-7619-DAP-17875

PCR Amplification

The 16S rRNA gene was amplified by PCR with universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′) (10). In a 25 μl reaction mixture, 1.5 μl of DNA, 12.5 μl of Green Master Mix PCR (Promega, USA), 1 μl of each primer, and nuclease-free water were used for PCR amplification. Thirty-five cycles of denaturation for 45 seconds, annealing at 55°C for 45 seconds, extension at 72°C for 45 seconds, and final extension at 72°C for 7 minutes were performed after the initial denaturation at 95°C for 3 minutes. A 1.5% agarose gel electrophoresis was used to separate the PCR products.

Sequencing

The Sanger sequencing method was used to sequence the amplified PCR products (25 μL) and 50 μL of primers, which were delivered to Macrogen (Seoul, South Korea). BioEdit software and NCBI's BLAST program were used to evaluate the resultant sequences. Homologous sequences were found by comparing them to the NCBI GenBank database. To evaluate the robustness of the tree topology, the phylogenetic tree was visually shown using the Molecular Evolutionary Genetics Analysis (MEGA) software version 6.0 and the neighbor-joining procedure with 1,000 bootstrap replicates. The acquired 16S rRNA sequences were added to the NCBI GenBank database with the accession numbers listed below: (OM943844.1, OM943845.1, OM943846.1, OM943847.1, OM943848.1)

Results

The results presented in this study were based on analyses of data from a total of 50 children with autism. The findings indicated a higher risk of autism in males (40, 80%) compared to females (10, 20%).
To identify the 16S rRNA gene, 50 samples were subjected to PCR analysis. PCR analysis showed that 10% (5/50) of the samples had positive *Clostridium ventriculi*

10.15789/2220-7619-DAP-17875

findings, distributed as 3 males and 2 females. All control group samples were negative for this bacterium.

Certain genetic alterations in *Clostridium ventriculi* are revealed by the 16S rRNA gene sequencing analysis. At nucleotide positions 63 and 43, respectively, these alterations consist of one transition (C to T) and one transversion (T to A). Furthermore, at nucleotide positions 93 and 129, there is one transversion (G to C) and one transition (A to G). Additionally, there are three transversions (A to C) at nucleotide positions 157, 195, and 207, as well as a transition (T to A). Moreover, there is one transition (C to T) at nucleotide position 227, and one transition (C to T) and one transversion (C to A) at nucleotide positions 306 and 328.

Analysis of the GenBank revealed a segment of the 16S rRNA gene that has 99% similarity with the 16S rRNA gene sequence in NCBI, as shown in Table 1.

The sequences with the highest degree of similarity and the most extensive overlap were discovered by comparing isolates acquired from dialysis patients. A neighbor-joining tree was created for the phylogenetic analysis. The genetic relatedness between Iraqi isolates and isolates from various regions worldwide is illustrated using the phylogenetic tree. According to hierarchical cluster analysis, there are multiple clusters. The main cluster is separated into several subgroups. Many global isolates showed a high degree of similarity to the Iraqi isolates studied in this research, as shown in Figure 1.

Discussion

Clostridium ventriculi is a species of bacteria that has been found to exist at higher levels in the gastrointestinal tracts of individuals with autism spectrum disorder (ASD) [25]. The link between *Clostridium ventriculi* and autism has garnered attention, with scientists exploring how gut bacteria affect the behavior and symptoms of children with autism in Iraq.

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10.15789/2220-7619-DAP-17875

De Angelis' study used the 16S rRNA gene, a marker for bacterial species, to find *Clostridium ventriculi* in autistic people [9]. The findings of the study may be important in determining how *Clostridium ventriculi* affects the gut microbiota of autistic individuals. Research into how gut bacteria may impact the behavioral elements of autism may be prompted if a consistent connection between this bacterium and the disorder is proven [9]. ASD symptoms may be exacerbated by imbalances, such as an excess or deficiency of specific bacteria, as studies have revealed changes in the gut microbiota of people with ASD [30, 11].

Numerous studies have shown that individuals with autism spectrum disorder (ASD) had greater levels of *Clostridium* than control groups. Additionally, according to these studies, *Clostridium* may create neurotoxins that could affect how ASD develops [10, 3].

The 16S rRNA gene offers several compelling advantages for *Clostridium ventriculi* phylogenetic analysis. Despite its conserved nature, the 16S rRNA gene has both conserved and variable sections that aid in species differentiation. This dual nature makes it feasible to draw evolutionary connections between *Clostridium ventriculi* and other genus members. Even in preserved areas, research has shown that there is enough variation to effectively resolve phylogenetic relationships [33, 17]. A strong basis for comparison is provided by the large collection of 16S rRNA sequences. Because there are several sequences from related *Clostridium* species, researchers may accurately position *C. ventriculi* into the wider clade, increasing the validity of phylogenetic findings [14, 7].

The distribution of the Iraqi samples throughout several phylogenetic tree branches indicates genetic variety. According to the study of relationships, there may be variance within this group, as certain Iraqi isolates (such as OM943844.1 and OM943845.1) have closer genetic distances than others (like OM943846.1). Variations in environmental adaptability and niche specialization within the gastrointestinal tract or other environments may be the cause of the observed genetic

10.15789/2220-7619-DAP-17875

variety among Iraqi *Clostridium ventriculi* isolates, as shown by their dispersion across the phylogenetic tree [2].

The distribution of *Clostridium ventriculi* isolates from Iraq over various branches of the phylogenetic tree indicates genetic variation among these isolates. Certain Iraqi isolates (like OM943844.1 and OM943845.1) form close clusters with little genetic separation, while others (like OM943846.1) split out more widely, suggesting variation within the group. Environmental influences, host-specific adaptations, or evolutionary divergence throughout time could all be responsible for this diversity. Bacterial phylogenetic studies frequently emphasize the ways in which ecological niches and geographic isolation affect genetic variation within species [28, 24].

The spread of this ancestor, combined with elements like genetic drift, a small population size, or a lack of selective forces that promote diversity, may be the cause of the genetic similarity shown in the isolates from Iraq. Consequently, the strains' genetic diversity might be limited [18, 20].

A cluster of isolates from the Czech Republic, Saudi Arabia, Japan, the UK, Norway, Ireland, and Russia was shown to have a tight genetic link based on the phylogenetic tree. Furthermore, while some isolates from Iraq constitute a distinct branch, others are detected inside this cluster, indicating a more distant relationship. The intricate connections between *C. ventriculi* strains from various geographic regions are further highlighted by the appearance of a single isolate from China on a separate branch. The genetic similarity between bacterial strains from different nations can be explained by a combination of horizontal gene transfer, environmental influences, and core genome historical movement patterns. These elements reinforce the intricate relationships discovered in bacterial phylogenetics, which allow

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populations from many geographic areas to exhibit striking genetic similarities [23, 28, 34].

Conclusion

The investigation of *Clostridium ventriculi* and autism spectrum disorder (ASD) demonstrates the increasing interest in the gut-brain-microbiota axis and its possible connection to neurological conditions. Research has shown that people with ASD have notable changes in the composition of their gut microbiota, including higher concentrations of *Clostridium ventriculi*. Through processes including neurotoxic generation and intestinal dysbiosis, which can affect behavior and brain function, this bacterium may exacerbate symptoms of ASD.

The genetic diversity of *Clostridium ventriculi* isolates from Iraq and other places has been better understood thanks to phylogenetic analysis using the 16S rRNA gene. The differences between isolates could be the result of evolutionary divergence, host-specific variables, or environmental adaptations. These results imply that the genetic composition of bacterial strains is influenced by ecological and geographic factors.

Even though the link between *Clostridium ventriculi* and ASD is encouraging, more investigation is required to prove causation and comprehend the underlying mechanisms. If verified, this connection may pave the way for treatment measures that target gut bacteria in an effort to reduce symptoms of ASD. All things considered, the research emphasizes how critical it is to comprehend how the gut microbiota contributes to neurological conditions and the promise of microbiome-based therapies.

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10.15789/2220-7619-DAP-17875

ТАБЛИЦЫ

Table 1. Type of polymorphisms of 16S rRNA gene.

No. Of sample	Type of substitution	Location	Nucleotide change	Reference Sequence	Sequence ID submission
1	Transvertion	43	T\A	ID: MG733966.1	ID: OM943844.1
	Transition	63	C\T		
2	Transition	93	A\G	ID: MG733966.1	ID: OM943845.1
	Transvertion	129	G\C		
3	Transvertion	157	A\C	ID: MG733966.1	ID: OM943846.1
	Transvertion	195	A\C		
	Transvertion	207	T\A		
4	Transition	227	C\T	ID: MG733966.1	ID: OM943847.1
5	Transition	306	C\T	ID: MG733966.1	ID: OM943848.1
	Transvertion	328	C\A		

Note: Abbreviations: T: Thymine, A: Adenine, C: Cytosine, G: Guanine.

ISSN 2220-7619 (Print)
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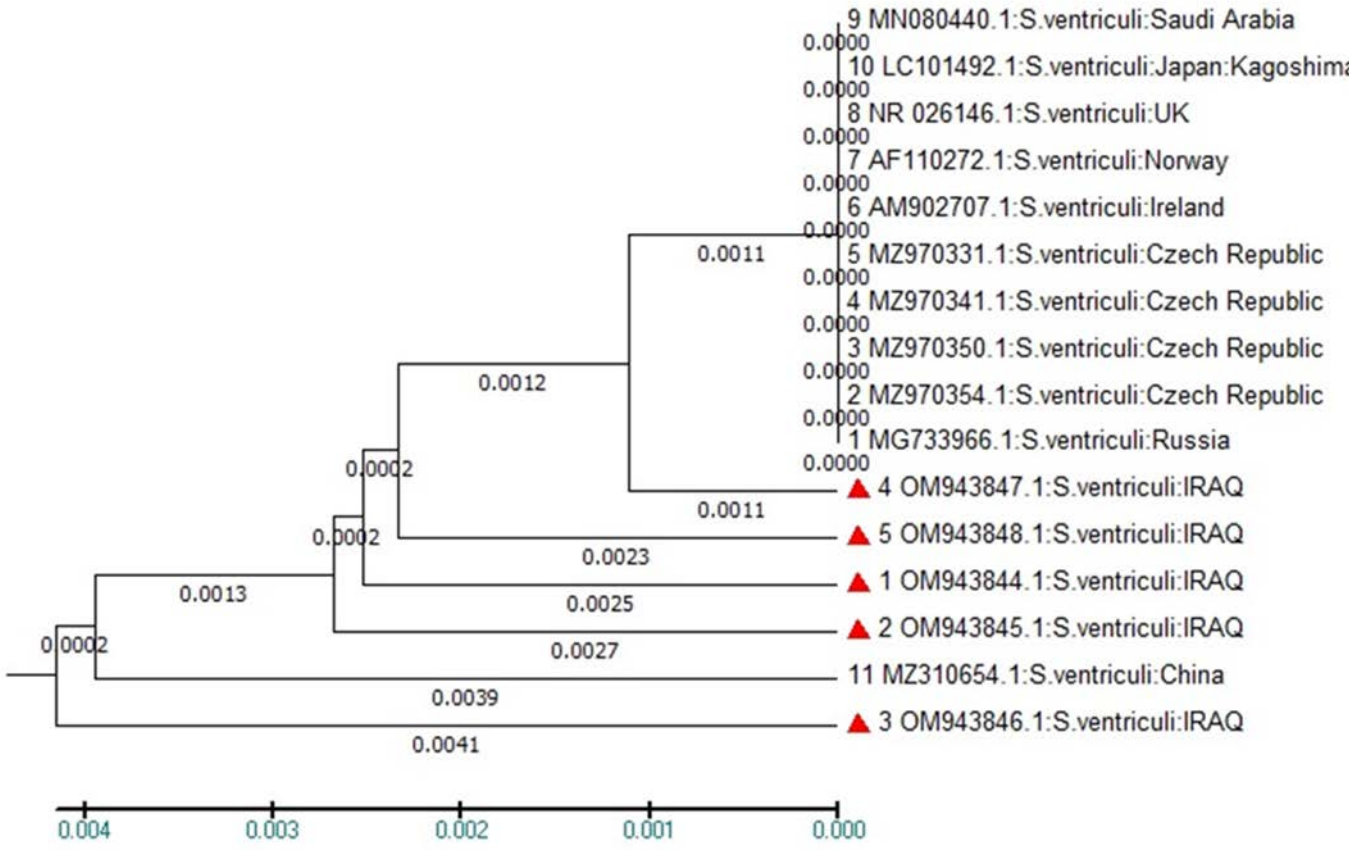
РИСУНКИ

Figure 1. Neighbor-joining tree *Clostridium ventriculi* of 16S rRNA gene. Red triangles indicate the positions of the Iraqi isolates within the phylogenetic tree.



ISSN 2220-7619 (Print)
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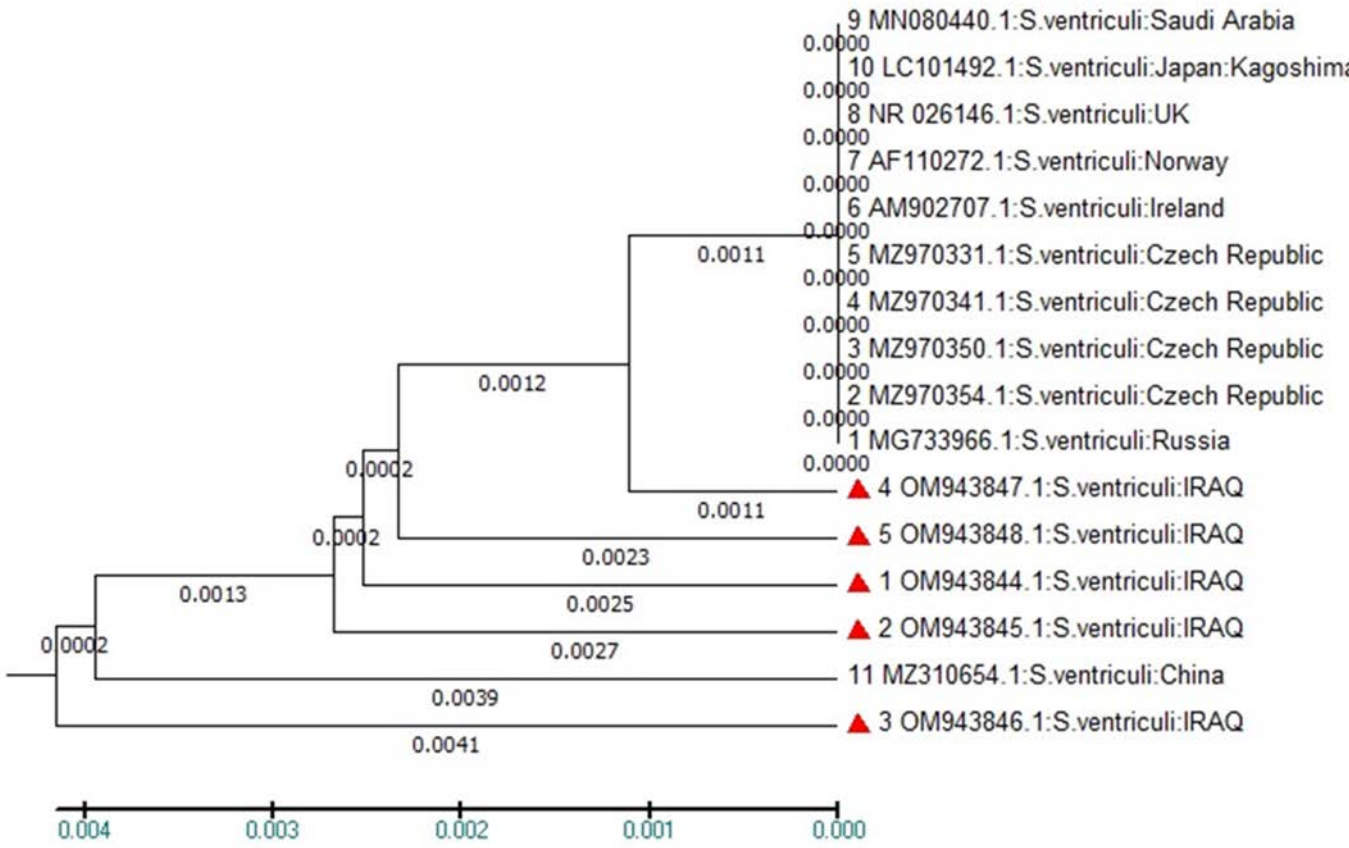
10.15789/2220-7619-DAP-17875

Figure 2. Neighbor-joining tree *Clostridium ventriculi* of 16S rRNA gene.



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10.15789/2220-7619-DAP-17875

ТИТУЛЬНЫЙ ЛИСТ_МЕТАДАННЫЕ

Блок 1. Информация об авторе ответственном за переписку

Dr. Husam Hussein Lazim, Ph.D. Microbiology, lecturer;

Ibn Sina University of Medical and Pharmaceutical Sciences, Baghdad, Iraq;

telephone: 009647801612798;

e-mail: hlazim@ibnsina.edu.iq

Блок 1. Информация об авторе ответственном за переписку

Доктор Хусам Хусейн Лазим, доктор философии по Микробиологии, преподаватель;

Университет медицинских и фармацевтических наук Ибн Сины, Багдад, Ирак;

телефон: 009647801612798;

электронная почта: hlazim@ibnsina.edu.iq

Блок 2. Информация об авторах

Dr. Alaa Hussein Hasan, Ph.D. Microbiology, Lecturer;

Ibn Sina University of Medical and Pharmaceutical Sciences, Baghdad, Iraq

Блок 2. Информация об авторах

Доктор Алаа Хусейн Хасан, доктор философии по Микробиологии, преподаватель;

Университет медицинских и фармацевтических наук Ибн Сины, Багдад, Ирак

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Блок 3. Метаданные статьи

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ВЫЯВЛЕНИЕ И ФИЛОГЕНЕТИЧЕСКИЙ АНАЛИЗ Clostridium VENTRICULI У ДЕТЕЙ С АУТИЗМОМ

Сокращенное название статьи для верхнего колонтитула:

CLOSTRIDIUM VENTRICULI IN AUTISTIC CHILDREN: A GENETIC PERSPECTIVE

CLOSTRIDIUM VENTRICULI У ДЕТЕЙ-АУТИСТОВ: ГЕНЕТИЧЕСКИЙ АНАЛИЗ

Keywords: *Clostridium ventriculi*, autism spectrum disorder, phylogenetic analysis, 16S rRNA gene.

Ключевые слова: Clostridium ventriculi, расстройство аутистического спектра, филогенетический анализ, ген 16S рРНК.

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