

Metagenomic Next-Generation Sequencing Identifies Enterovirus C and Elizabethkingia meningoseptica in Pediatric Central Nervous System Infections in Nepal

Nishan Katuwal

Molecular and Genome Sequencing Research Lab, Dhulikhel Hospital

Aastha Shrestha

Molecular and Genome Sequencing Research Lab, Dhulikhel Hospital

Dilli Ram Kafle

Nobel Medical College Teaching Hospital

Babita Khanal

Nobel Medical College Teaching Hospital

Sudichhya Tamrakar

Center for Infectious Disease Research and Surveillance, Dhulikhel Hospital

Sony Shrestha

Center for Infectious Disease Research and Surveillance, Dhulikhel Hospital

Sabin Bikram Shahi

Center for Infectious Disease Research and Surveillance, Dhulikhel Hospital

Dipesh Tamrakar

Center for Infectious Disease Research and Surveillance, Dhulikhel Hospital

Sony Shakya Shrestha

Center for Infectious Disease Research and Surveillance, Dhulikhel Hospital

Rajeev Shrestha

`rajeev.shrestha@kusms.edu.np`

Center for Infectious Disease Research and Surveillance, Dhulikhel Hospital

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Abstract

Background

Central nervous system (CNS) infections, including encephalitis, meningitis, and meningoencephalitis, remain major causes of morbidity and mortality, yet their etiologies are diverse and remain undiagnosed using conventional diagnostic methods. Metagenomic next-generation sequencing enables unbiased detection of diverse pathogens and may support etiological diagnosis of CNS infections. This study investigated infectious etiologies of suspected CNS infections in eastern Nepal using mNGS combined with clinical and laboratory data.

Methods

A prospective cross-sectional study was conducted among subjects with suspected encephalitis, meningitis, or meningoencephalitis presenting to a tertiary referral hospital in eastern Nepal between May and December 2023. Cerebrospinal fluid samples were collected for routine clinical investigations and mNGS. Nucleic acids were extracted, RNA and DNA libraries were sequenced on Illumina iSeq100.

Results

Of 200 enrolled subjects, *Elizabethkingia meningoseptica* was detected in eight pediatric subjects, while Enterovirus C was identified in nine and four pediatric subjects had both organisms. Phylogenetic analysis revealed that Enterovirus C clustered closely with Coxsackievirus-A24 strains previously reported from China, Pakistan, and India, while *E. meningoseptica* was closely related to strains reported from healthcare settings. Most pathogen-positive subjects occurred in infants and young children with meningitis or meningoencephalitis. Clinical and cerebrospinal fluid biochemical findings showed considerable overlap between bacterial and viral infections.

Conclusion

This study detected Enterovirus C and *E. meningoseptica* in pediatric CNS infections in Nepal, including cases of co-detection. These findings suggest that mNGS could complement conventional diagnostic approaches and depict the pathogen landscape associated with CNS infections.

Background

Encephalitis, meningitis, and meningoencephalitis are inflammatory neurological conditions with substantial global morbidity and mortality [1–3]. Nepal's acute encephalitis syndrome incidence far exceeds regional estimates. The cases of acute encephalitis syndrome (5.23/100,000) in Nepal are

estimated to be more than 12 times higher than in India (0.42/100,000) [4, 5]. However, population-based research on incidence of AES has not been conducted in Nepal.

In Nepal, Japanese Encephalitis (JE) has historically been a dominant etiology, with its endemic area expanding from terai to hilly and mountainous districts [6–8]. However, JE accounts for only ~ 15% of the cases while 37–75% of cases remain etiologically undiagnosed. [8–13] Further, emerging evidence points to diverse viral, bacterial and fungal etiologies with overlapping clinical presentations, complicating diagnosis [1, 9, 14, 15]. The epidemiology of these pathogens also varies geographically, with recent investigative and surveillance studies reporting non-JEV etiologies in Nepal and India [15–17]. The current surveillance approaches are either limited by dependence of prior genomic knowledge of the pathogen or dependent on large commercial panels, which do not always include pathogen of epidemiological relevance [18, 19].

Metagenomic next-generation sequencing (mNGS) provides an unbiased sequencing approach that capable of detecting viral, bacterial, fungal, and parasitic pathogens, without the need of knowledge on the causative agents [15, 16]. This makes mNGS particularly well suited to settings like Nepal, where the etiological landscape of CNS infections remains poorly characterized and likely includes pathogens not captured by conventional syndromic panels. Thus, this study applies unbiased metagenomic next-generation sequencing combined with investigation of clinical and laboratory parameters to understand etiologies of encephalitis, meningitis and meningoencephalitis.

Methods

Study design

This cross-sectional study included prospective enrollment of 200 subjects suffering from acute encephalitis, meningitis and meningoencephalitis. The subjects (of all ages) were enrolled from the adult and pediatric outpatient neurological department of Nobel Medical College Teaching Hospital, Biratnagar, Nepal, from May to December 2023.

Sample and Data Collection

The enrollment involved collection of cerebrospinal fluid (CSF), in RNA/DNA Shield (Zymo Research, Cat: R1200) with questionnaire (collected in KoboToolbox) including clinical metadata (onset of disease, medication, cell counts, glucose level, laboratory investigation data) and demographic information, after written consent. Each of the samples were given unique study specific IDs. The collected samples were stored at -20°C until transported to Dhulikhel Hospital, in cold chain. Further, process controls were collected from the skin and procedure area every week during the enrollment period.

Nucleic Acid Extraction and Next Generation Sequencing

The nucleic acid (RNA and DNA) was extracted from cerebrospinal fluid using Quick DNA/RNA Pathogen MiniPrep Kit (Zymo Research, Cat: R1042). This was followed by library preparations using NEBNext Ultra II RNA Library Prep Kit (New England Biolabs. Cat: E7770L) for RNA Library Preparation and NEBNext Ultra II FS DNA Library Prep Kit (New England Biolabs. Cat: E7805L) for DNA Library Preparation. The prepared libraries were checked for concentration (100-120pM), primer dimers and library size using Qubit HS dsDNA Assay (Invitrogen. Cat: Q32851) and High Sensitivity D5000 HS Assay from Agilent TapeStation 4150 (Agilent Technologies. Cat: 5067–5593). The libraries included negative extraction control and negative library preparation controls were included in each extraction and library preparation batch. The quality checked and passed libraries were loaded in Illumina iSeq100.

Genomic Analysis

The genomic analysis was performed on the CZID using the Illumina mNGS Pipeline v8.3, with background created from process controls. The heatmaps were generated using the threshold of NT rPM (nucleotide reads per million) ≥ 10 and NT L (alignment length in base pairs: length of the aligned sequence) ≥ 50 . The background was created using process control that included negative extraction control, negative library preparation control, skin swabs from subjects and swabs of procedure room.

Results

Sample Collection and Subject Demography

The cerebrospinal fluids were collected from 190 subjects, while remaining procedures were either cancelled or there was not enough CSF available after routine investigation. The enrolled subjects aged between 3 months to 75 years, with mean age of 23.6 years, with 56% being female. The subjects were mostly from Morang District (34%) in Nepal followed by Sunsari (17%) and Jhapa (9.5%). There were a total of 12 subjects (6%) from India (with 3 among them from bordering region of Bihar). 91% of the enrolled subjects did not have travel history, while 4% had travel history within Nepal. Of the remaining 5% (n = 10), 4 were Nepalese citizens and had travel history to India, while other 6 were Indian citizens with travel history to Nepal, including the visit for treatment. Of all subjects, 19 (9.5%) reported mosquito bites, while none reported contact with ticks. More than half (54.5%) of the subjects lived near pig or other animal farms, with 48.5% (97), 34.0% (68) and 17.5% (35) residing in peri-urban, rural and urban areas respectively. Of the cases below 18 years, 46 (43.09%) subjects had taken vaccination against JE while, for the cases above 18 years, only 3 (3.2%) subjects had history of vaccination. The details of subject demography can be found in Table 1.

Table 1
Demographic Information in adult (> 18 years) and underaged (< 18 years) subjects

Demographic Characteristic	Population Type	Frequency	Frequency
		in adult subjects (n, %)	in underaged subjects (n, %)
Educational Background	Not formally educated but literate	18	0
	Higher Secondary (+ 2)	10	0
	Illiterate	41	0
	Secondary	18	11
	Bachelors	4	0
	Primary	1	29
	Do not want to answer	1	0
Occupation	Agricultural Field	53	2
	Student	15	50
	Do not want to answer	4	0
	Unemployed	3	0
	Homemaker	1	0
	Wage Earner	1	0
	Retired	1	0
	Others	15	3
Family Income (in Nepalese Rupees)	10001–20000	4	10
	20001–30000	35	48
	40001–50000	2	3
	30001–40000	19	20
	50001–60000	2	0
	Do not want to answer	31	25

mNGS of DNA Library

Out of 122 CSF samples (57 adults and 65 children), DNA libraries for 30 did not pass quality filters. *Elizabethkingia meningoseptica* was detected in 8 underaged (< 18 years) subjects (NC_003, NC_009, NC_011, NC_014, NC_019, NC_020, NC_022, NC_023) with variable rPM (Fig. 1). All of these subjects were from Nepal.

Out of these, partial coverage was observed in NC_009 (Fig. 2), while no coverage visualization was available for remaining libraries.

mNGS of RNA Library

Out of 122 CSF samples, RNA libraries for 95 RNA passed quality filters and were evaluated. *Enterovirus C* was detected in 9 underaged (< 18 years) subjects (NC_003, NC_006, NC_009, NC_010, NC_013, NC_016, NC_018, NC_019, NC_022) with different rPM (Fig. 3). All of these subjects were from Nepal.

Out of these, complete contig coverage (Fig. 4) was observed in NC_009 (68.4x, 99.8%), NC_016 (64.4x, 99.8%) and NC_022 (67x, 99.8%); partial coverage (Fig. 5) in NC_003, NC_006, NC_010, NC_013 and NC_019; and no coverage visualization due to unavailability of assembled contigs in NC_018.

Genomic Investigation

The *E. meningoseptica* strain identified in this study was closely related to strains G4120, 2022CK-00380. (Fig. 6) These strains were from unpublished studies that evaluated samples from healthcare settings.

The Enterovirus C strain identified in this study was closely related to strains CA24v, AHC and RMRC-DIB-1311. (Fig. 7) These strains were from Northeast India (Assam), Pakistan and China. However, all of these Coxsackievirus A24 strains were isolated from conjunctival swabs.

Clinical Parameters

Most of the enrolled subjects had elevated WBC ($> 11 \times 10^9/L$). The CSF cell counts were highly varying from < 5 to 815 WBC/ml. In subjects NC_003, NC_009, NC_011, NC_014, NC_019, NC_020, NC_022 and NC_023 with *E. meningoseptica* infection, age ranged from 4 months to 15 years, with NC_011 (age 3 months), NC_020 (8 years) and NC_023 (3 years 9 months) also having past history (within past one month) of neurological disorders such as global developmental delay with seizure disorder, severe headache and meningitis respectively. The primary diagnosis for these three subjects (NC_011, NC_020 and NC_023) was meningoencephalitis, meningitis, and meningoencephalitis, respectively. For NC_009 (aged 4 months) which had partial coverage, meningitis was stated as primary diagnosis with symptoms such as fever and neck stiffness and colic abdomen. These subjects were under no medication and were admitted in general ward (Table 2). The CSF gram stain and culture showed clear samples (pus cells in some samples) and no microbial growth.

In subjects NC_003, NC_006, NC_009, NC_010, NC_013, NC_016, NC_018, NC_019, and NC_022 with Enterovirus C infection, age ranged from age 3 months to 15 years, with NC_010, NC_016 and NC_018 having past history (8 days, 5 months and 8 month ago respectively) of neurological disorders such as meningitis with sepsis, febrile convulsions and meningitis respectively. The primary diagnosis for these subjects was meningitis, meningoencephalitis with febrile seizures and meningitis respectively. For NC_009 (aged 4 months), NC_016 (aged 12 months) and NC_022 (aged 12 months) which had complete coverage, meningitis (NC_009) and meningoencephalitis (NC_016 and NC_022) was stated as primary diagnosis with symptoms such as fever, seizure, vomiting, neck stiffness and colic abdomen. These subjects were prescribed with antibiotics (all except NC_009) and combination of antiviral and antibiotic (NC_003), while all subjects except NC_009 and NC_0022 were admitted to the intensive care unit. The CSF cell counts for all these subjects ranged from < 5 to 15 WBC/ml. The average CSF Glucose and Protein among Enterovirus infected was 64.7 and 47.2 mg/dL while for *E. meningoseptica* was 75.6 and 56.0 mg/dL respectively. (Table 2) Similarly, in subjects with infections from *E. meningoseptica* and Enterovirus C, the lowest leukocyte count was 8,100 and 3,100 respectively.

Table 2
Summary of laboratory parameters and pathogens detected for subjects with *E. meningoseptica*, Enterovirus or both.

Subject	Organism Detected	CSF Glucose (mg/dL)	CSF Protein (mg/dL)	Leukocyte Counts
N_C_003	Enterovirus C, <i>E. meningoseptica</i>	64	56	8,600
N_C_006	Enterovirus C	49	65	12,100
N_C_009	Enterovirus C, <i>E. meningoseptica</i>	76	46	13,400
N_C_010	Enterovirus C	60	45	3100
N_C_011	<i>E. meningoseptica</i>	60	49	13,600
N_C_013	Enterovirus C	93	34	6,700
N_C_014	<i>E. meningoseptica</i>	57	60	8,100
N_C_016	Enterovirus C	80	59	14,500
N_C_018	Enterovirus C	57	22	15,200
N_C_019	Enterovirus C, <i>E. meningoseptica</i>	52	49	12,300
N_C_020	<i>E. meningoseptica</i>	200	79	8,100
N_C_022	Enterovirus C, <i>E. meningoseptica</i>	51	49	14,200
N_C_023	<i>E. meningoseptica</i>	45	60	9,900

Discussion

Subject Demography

The demographic profile of the study population is difficult to compare with previous studies because comparable cohorts from Nepal are limited. Published studies have either screened only adults (above 16 years) or only children (1–14 years) [8, 20], whereas our study included participants from both age groups. The subjects in our study were predominantly from the densely populated districts of eastern Terai that are served by this tertiary referral hospital. Additionally, Morang and Jhapa had a high number of subjects, which were also reported in 2012 [9]. The findings from subjects of neighboring Indian states, particularly Bihar, shows the shared epidemiology of encephalitic diseases, emphasizing need for coordinated cross-border surveillance and control strategies [9]. Furthermore, the low reported coverage of JE vaccination, in our study compared to national average [21, 22], highlights an important public

health concern particularly given the continued endemicity of the disease in eastern Nepal and recent reports of deaths. [23]

The predominance of adults engaged in agriculture and modest socioeconomic status may also influence exposure risk through occupational and environmental factors [24, 25] and affecting healthcare access and health-seeking behavior, warranting further investigation.

Whole Genome Sequencing

E. meningoseptica was first described as a group of unclassified bacteria associated with meningitis in infants and has also been associated with neonatal meningitis, sepsis and nosocomial infections in immuno-compromised populations, although rare [26]. Several studies have reported this pathogen to infect adults and children causing meningitis, while being found primarily in blood as well as cerebrospinal fluid [27–29]. This is similar to the findings of our study, where most of the infected subjects were children and had primary diagnosis of meningitis.

The *Enterovirus C* was closely related to isolates from outbreaks in northeast India, Pakistan and China. However, these strains were isolated from conjunctival swabs, therefore inferring conjunctivitis infections instead of meningitis or meningoencephalitis or acute encephalitis [30–32]. There are 23 types of Enterovirus C that cause a range of diseases such as acute respiratory infections, myocarditis, acute gastroenteritis, acute hemorrhagic conjunctivitis, encephalitis, meningoencephalitis, rhombencephalitis and meningitis among others [33, 34]. Nonetheless, some other studies have observed Enterovirus C, particularly strain CVA24, to be a causative agent of aseptic meningitis [33, 34]. In our findings, the subjects infected with Enterovirus C also had meningitis and meningoencephalitis, and were mostly children.

Clinical Parameters

The clear appearance and absence of microorganisms on gram stain and sterile bacterial cultures despite symptomatic subject suggest that routine bacteriological diagnostics alone are insufficient. Further, CSF biochemical findings showed overlap between viral and bacterial groups, with variability in CSF glucose and protein levels and inconsistent leukocyte counts. Notably, some subjects with bacterial pathogens did not show classical hypoglycorrhachia, while some viral subjects exhibited mildly elevated protein. For instance, subjects with infection from Enterovirus C had low CSF glucose and comparable protein values. Studies investigating *E. meningoseptica* have shown high CSF Protein (90mg/dL), which is comparable to the findings of this study (average 75.6mg/dL) [27]. The protein in CSF is higher in bacterial infections, due to increased number of replicating bacteria and is in range or is slightly higher in viral infections, which is similar to our findings [35, 36]. Further, CSF glucose is in normal range in most viral infections, similar to our findings [36]. However, the glucose level in bacterial infections is known to decrease, particularly in infants, due to glycolysis by both white cells and the pathogen as well as due to impaired CSF glucose transport [37]. However, this contrasts to the findings of our study. Nonetheless, studies have shown that CSF glucose levels are dependent on serum glucose levels, and in some infections, their performance remains inadequate for diagnosing bacterial meningitis [38, 39].

Limitations

This study was conducted at a single tertiary referral center over a limited enrollment period, which may not fully represent the epidemiology of related infections across Nepal. Second, the detection of microbial nucleic acids by mNGS does not always establish causality, particularly for organisms detected at low abundance or in co-infections, and confirmation by qPCR was not available for all positive findings in this study.

Conclusion

The detection of Enterovirus C and *Elizabethkingia meningoseptica*, including cases of co-infection, highlights the diverse pathogens associated with central nervous system infections. These findings could also inform the design of locally relevant multiplex pathogen panels by identifying etiologies not currently captured by routine or existing syndromic testing in our setting. Thus, when interpreted alongside with clinical and laboratory findings, metagenomic next generation sequencing could complement diagnosis, depict the pathogen landscape and provide genomic information to support pathogen surveillance, public health preparedness and further investigations of central nervous system infections in Nepal.

Abbreviations

AES Acute Encephalitis Syndrome

CNS Central Nervous System

CSF Cerebrospinal Fluid

CZ ID Chan Zuckerberg ID

DNA Deoxyribo Nucleic Acid

JE Japanese Encephalitis

mNGS Metagenomic Next Generation Sequencing

NCBI National Center for Biotechnology Information

NT Nucleotide

NT L Nucleotide Length

RNA Ribo Nucleic Acid

rPM Reads per million

Declarations

Ethics approval and consent to participate

Ethical approvals were obtained from Ethical Review Board at Nepal Health Research Council (ref: 64-2023) and Institutional Review Committee of Kathmandu University School of Medical Sciences (ref: 82/2023). Written informed consent was obtained for all participants of the study. In case of minors, the consent to participate was obtained from their parents/guardians. In case of illiterate subjects, the consent to participate was obtained using thumb impression along with consent from accompanying adult family members, as per national ethical guidelines. All procedures performed in study followed Helsinki's ethical standards and declaration.

Consent for publication

Not Applicable

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Author Contribution

N.K. wrote the main manuscript text and performed data analysis. N.K. and A.S. performed the primary investigation. N.K., A.S., S.T., S.S., and S.B.S. performed experimental studies and data acquisition. D.R.K. and B.K. performed clinical studies and data acquisition. D.T. and S.S.S. performed clinical studies and data analysis. R.S. designed and conceptualised the study, performed data analysis and final review of the manuscript. All authors reviewed the manuscript.

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Data Availability

The raw genomic data can be found in Sequence Read Archive, National Center for Biotechnology Information (NCBI), under BioProject PRJNA1499420.

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Figures

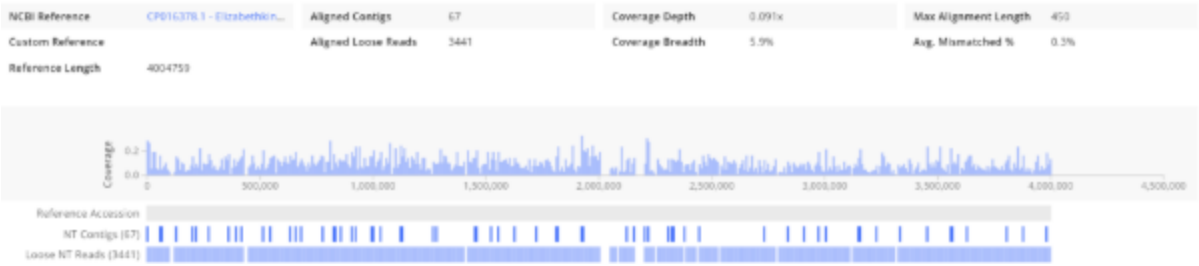


Figure 2

Coverage visualization of *E. meningoseptica* in samples NC_009 showing partial coverage width (5.9%).

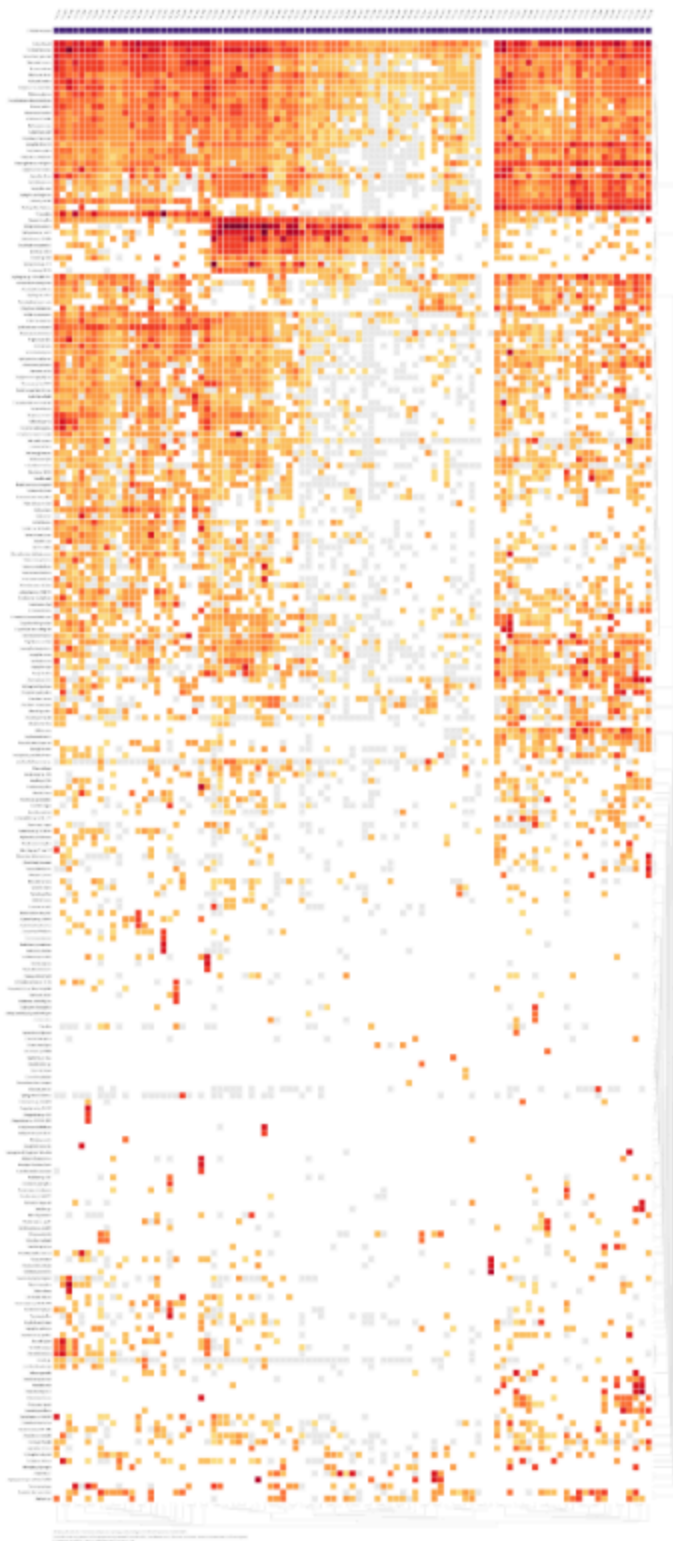
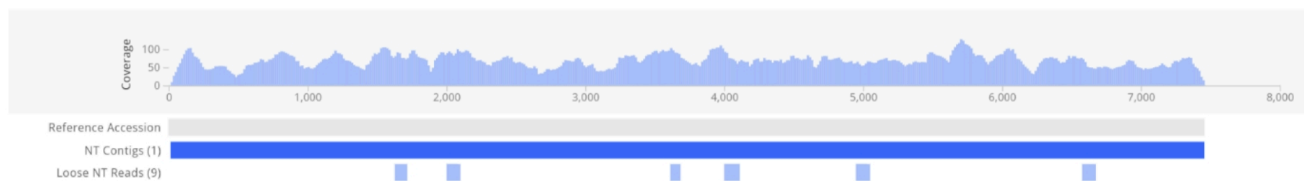


Figure 3

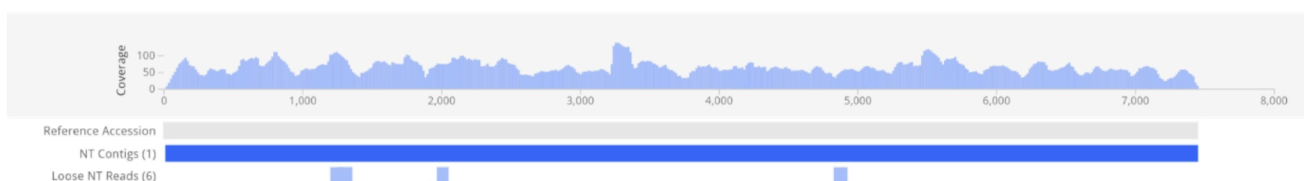
Heatmap depicting pathogens from sequencing of RNA libraries (n=95 CSF samples).

NCBI Reference	KR399988.1 - Cocksackievi...	Aligned Contigs	1	Coverage Depth	68.4x	Max Alignment Length	7444
Custom Reference		Aligned Loose Reads	9	Coverage Breadth	99.8%	Avg. Mismatched %	5.2%
Reference Length	7458						



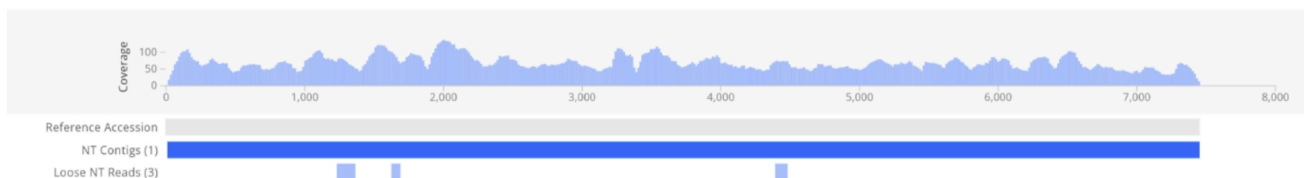
A)

NCBI Reference	KR399988.1 - Cocksackievi...	Aligned Contigs	1	Coverage Depth	64.4x	Max Alignment Length	7445
Custom Reference		Aligned Loose Reads	6	Coverage Breadth	99.8%	Avg. Mismatched %	5.4%
Reference Length	7458						



B)

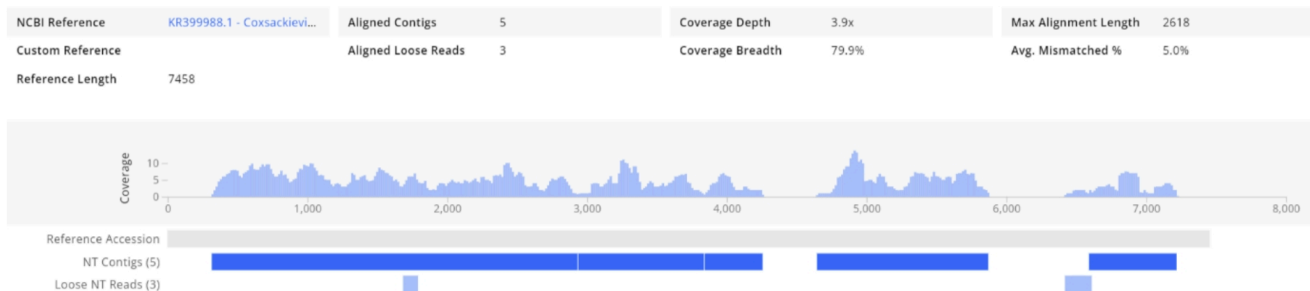
NCBI Reference	KR399988.1 - Cocksackievi...	Aligned Contigs	1	Coverage Depth	67x	Max Alignment Length	7445
Custom Reference		Aligned Loose Reads	3	Coverage Breadth	99.8%	Avg. Mismatched %	6.7%
Reference Length	7458						



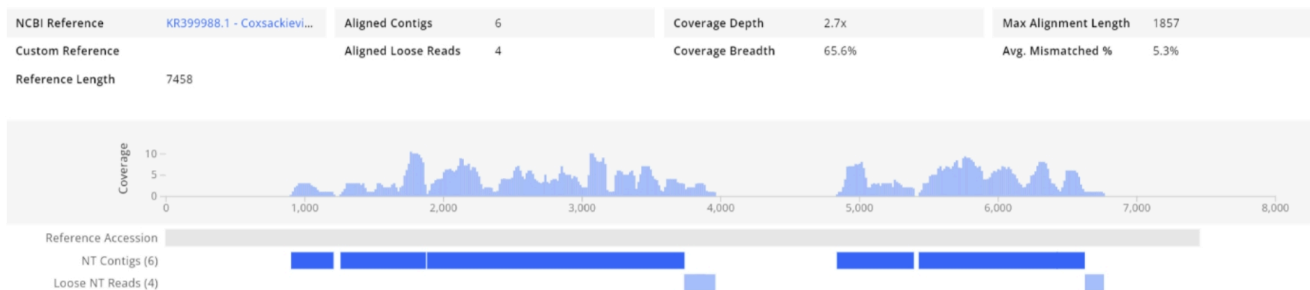
C)

Figure 4

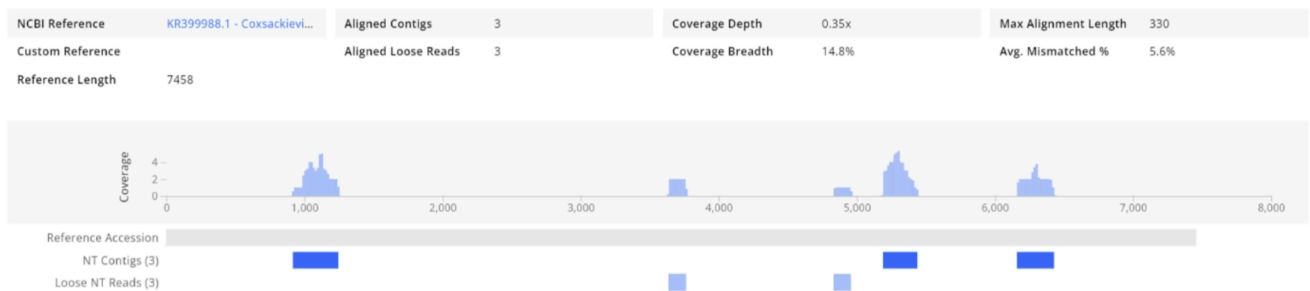
Coverage visualization of Enterovirus C in samples NC_009 (A), NC_016 (B) and NC_022 (C) showing complete coverage width (99.8%).



A)



B)



C)

Figure 5

Coverage visualization of Enterovirus C in samples NC_003 (A), NC_006 (B) and NC_013 (C) showing partial coverage.

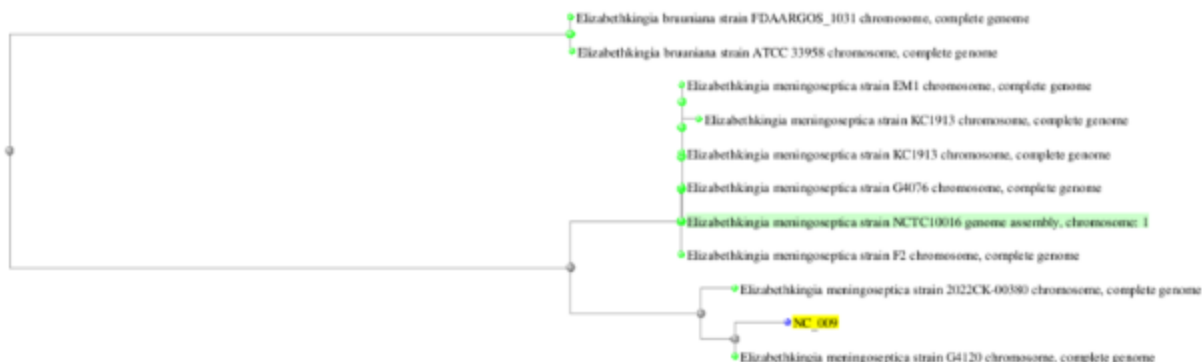
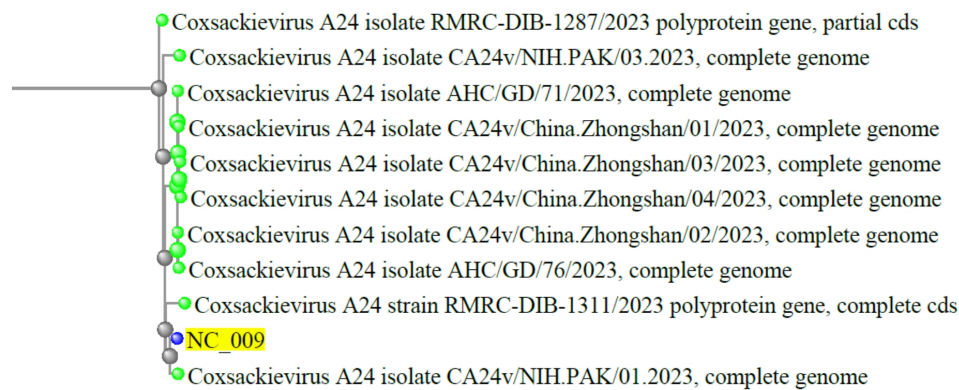
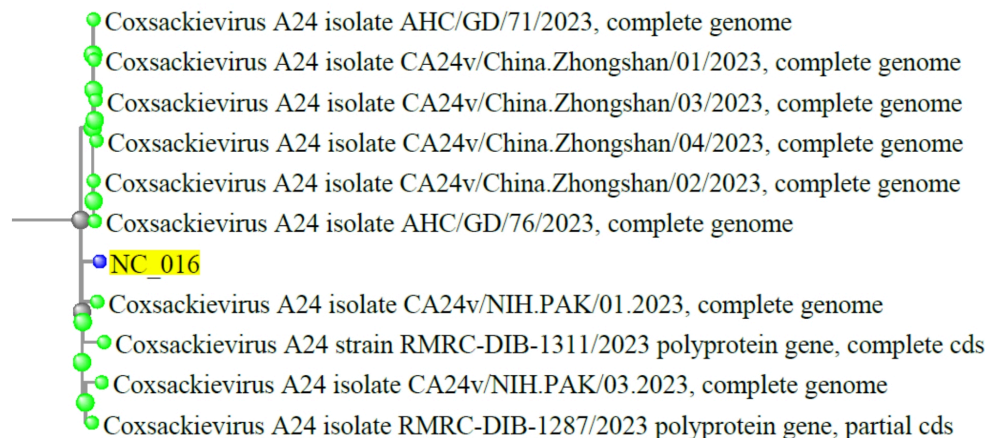


Figure 6

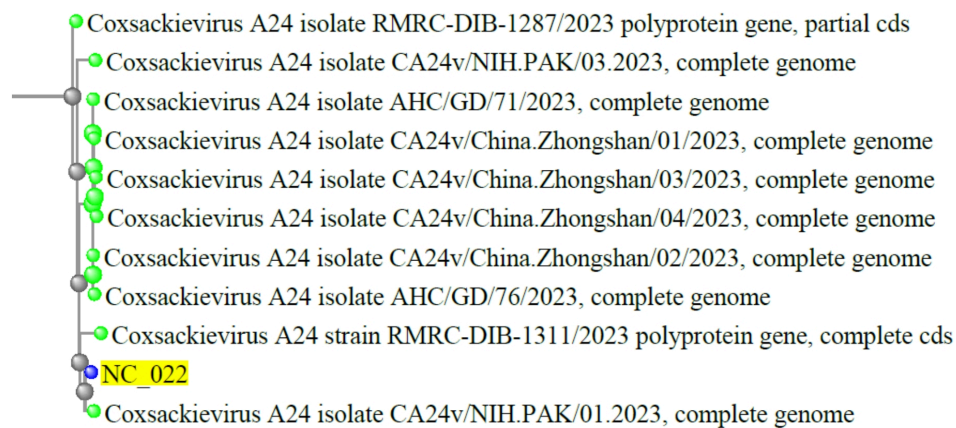
Pairwise alignment of largest contig (450bp) of NC_009 with other strains of *E. meningoseptica*.



A)



B)



C)

Figure 7

Pairwise alignment of contigs of NC_009 (7444bp), NC_016 (7445bp) and NC_022 (7445bp) with other strains of Enterovirus C.