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Identification of the Causative Pathogen in the 2023 Conjunctivitis Outbreak of Nepal Using Unbiased Metagenomic Next Generation Sequencing

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ABSTRACT

Background: In mid-2023, Nepal experienced a significant outbreak of conjunctivitis, affecting over 60% of outpatients in eye hospitals and prompting school closures. The outbreak, peaking in August, predominantly impacted children and individuals with compromised immunity. Clinical manifestations included sudden-onset redness, foreign body sensation, watery discharge, and occasional lid swelling. Most cases exhibited acute haemorrhagic conjunctivitis, with management involving ocular lubricants, personal hygiene, and topical antibiotics. This study from Himalaya Eye Hospital in Pokhara details the genomic epidemiology and clinical characteristics of conjunctivitis cases during the outbreak.

Methods: To understand the causative agents, conjunctival swabs from patients were subjected to unbiased metagenomic next-generation sequencing (mNGS) in Illumina iSeq100 at Dhulikhel Hospital Kathmandu University Hospital.

Results: This study revealed the presence of Enterovirus C (coxsackievirus strain A24) as the major pathogen responsible for the outbreak.

Conclusions: This study contributes valuable insights into the genomic diversity of conjunctivitis-associated viruses, highlighting the potential of mNGS in enhancing diagnostic capabilities and guiding public health responses.

Keywords: Conjunctivitis; enterovirus; outbreak.

INTRODUCTION

Conjunctivitis is the inflammation of the conjunctival tissue of the eye.[1,2] Viral conjunctivitis, constitute of 75% of the cases produces redness, irritation with watery discharge and occasional presence of preauricular adenopathy.[3,4] Around 90% of all viral conjunctivitis is caused by adenovirus.[5] The bacterial conjunctivitis is mostly caused by Staphylococcus spp., Haemophilus influenza, Streptococcus spp, Moraxella catarrhalis and other gram-negative bacteria.[6] Next generation sequencing approaches, have revealed diverse microbiome profile with some techniques being able of profiling to species level and is inherently unbiased and hypothesis-free.[7-10] Since July 2023, Nepal had an epidemic outbreak of conjunctivitis across the country. The surge was so high that more than 60% of the OPD patients in various eye hospitals of Nepal were filled with conjunctivitis cases and schools were shut down to prevent the community transmission. The cases mainly presented as pink eye, and viral origin was the top suspicion.[11]

METHODS

Himalaya Eye Hospital, Pokhara, Nepal experienced a conjunctivitis outbreak from mid-July until September 2023. This study was conducted under the tenets of declaration of Helsinki with the approval by Institutional Review Committee, Kathmandu University School of Medical Sciences (IRC, KUSMS). IRC-KUSMS approval number is 116/24. The written consents were

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taken from study participants. The participants were diagnosed as a case of conjunctivitis according to their symptoms and signs of redness, watering, foreign-body sensation, discharge and swelling of eyelids and conjunctiva. The conjunctival swabs were collected from the subjects (2-55 years) visiting HEH, as part of routine diagnosis, at the first day of ocular examination. These swabs were aliquoted in RNA/DNA Shield and transported (n=25) in cold chain to DHKUH. At DHKUH, the swabs were extracted for DNA and RNA (Zymo Quick DNA/RNA Pathogen Miniprep Kit, Cat: R0142) followed by subsequent DNA and RNA library preparation (New England Biolabs) for metagenomic next generation sequencing. The respective libraries were pooled, and quality checked by Agilent Tapestation and then loaded in Illumina iSeq100, with 4 million reads at 2x150 bp length. Additional process controls (extraction and library preparation) and sample controls (normal flora) were included as background model. The downstream metagenomic investigation was done by CZID (Illumina mNGS Pipeline v8.2), according to the read scores and Z-score. The phylogeny was made using maximum likelihood statistical method (in Mega X), Tamura-Nei model with nearest neighbours' interchange as the maximum likelihood heuristic method.

RESULTS

DDuring the outbreak season from July to September 2023, 49,544 subjects visited Himalayan Eye Hospital with conjunctivitis diagnosed (clinically) in 14,926 subjects (30.1%). The detailed case distribution during this period has been presented in Table 1.

Most of the cases presented with sudden onset of foreign body sensation with redness in one eye followed by involvement of the other eye in 1-2 days. There was presence of watery discharge, minimal pain, occasional lid swelling but prominent redness in some part of conjunctiva suggestive of acute haemorrhagic conjunctivitis. The management were done with the use of ocular lubricants, care of personal hygiene and topical antibiotics as per need. Among the conjunctival swabs received at DHKUH, samples from 22 subjects were evaluated at DHKUH using unbiased metagenomic next generation sequencing. As seen in figure 1, most of the samples had Cutibacterium acnes and Escherichia coli. We have also found this organism as part of healthy conjunctival flora, as per our previous studies (unpublished data). Other organisms observed that could likely cause conjunctivitis, were Micrococcus luteus and Sphingomonas spp. Enterovirus C (coxsackievirus strain A24) was seen in total of 10 samples (Figure 2), while four samples (S3, S9, S16, S21) had coverage width >95% for A24 strain, as seen in figure 3. The phylogenetic analysis of the consensus Enterovirus C genomes showed variation from other reported ocular infections associated coxsackievirus (from studies in China, Japan and France). Additionally, samples S3, S6 and S9 slightly had some common genomic regions with coxsackieviruses isolates from France.

(Mega X), Tamura-Nei model with nearest neighbours' interchange as the maximum likelihood heuristic method.

Table 1. Case distribution during the conjunctivitis outbreak from July-September 2023, in Himalaya Eye Hospital, Pokhara.

S. N.	Time Period	Total number of patients	Total number of conjunctivitis cases	Male conjunctivitis cases/Total male patients	Female conjunctivitis cases/Total female patients
1	15 July to 31 July 2023	11,140	580 (5.2%)	323/4,793	257/6,347
2	1 August to 31 August 2023	24,922	12,561 (50.4%)	6,727/12,146	5,825/12,770
3	1 September to 30 September 2023	13,482	1,785 (13.2%)	911/6,051	874/7,430

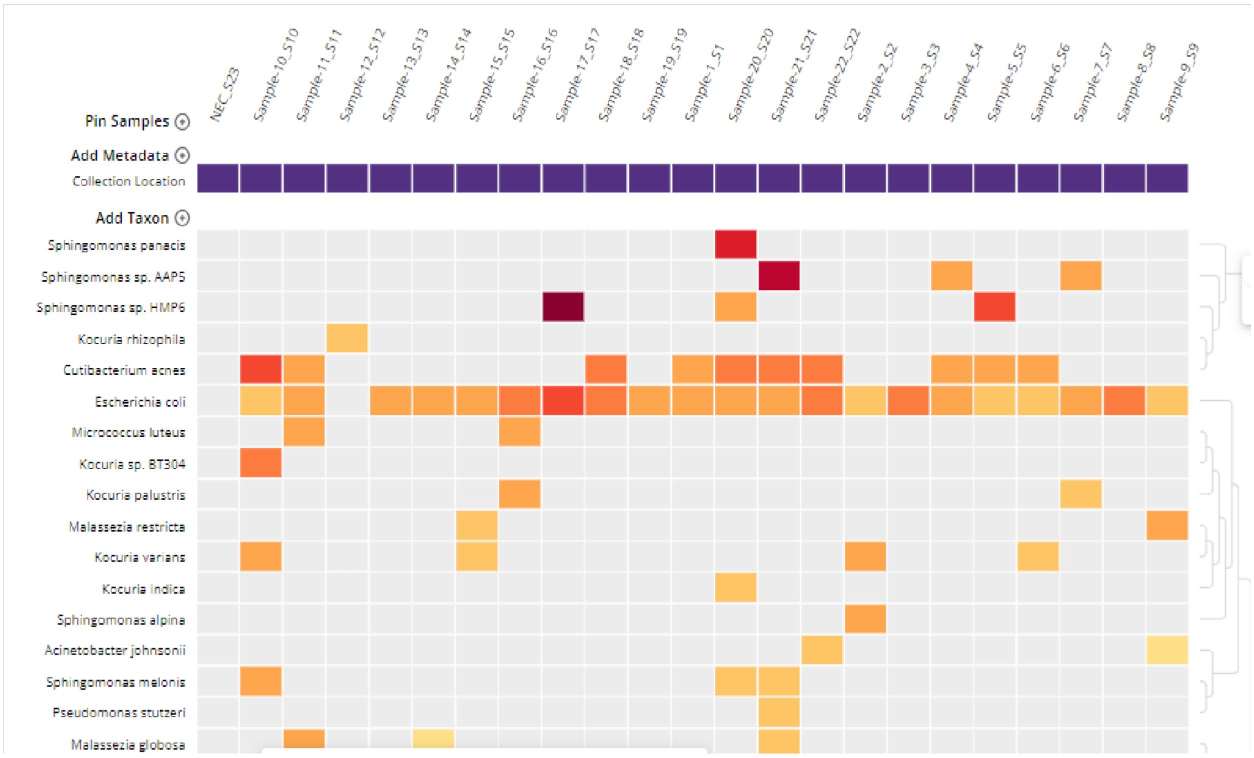


Figure 1: Heatmap of libraries for S1 to S22: The organisms (at the genus level) that were seen in the samples are shown on x axis, while the names of the samples are on the y axis. NEC in the figure stands from Negative Extraction Control, while NLC (Negative Library Control) did not enough reads to be seen in the heatmap. This heatmap used the background model from conjunctival samples of healthy controls with threshold of NT rPM (nucleotide reads per million) >=10 and NT L (alignment length in basepairs: length of the aligned sequence)>=50

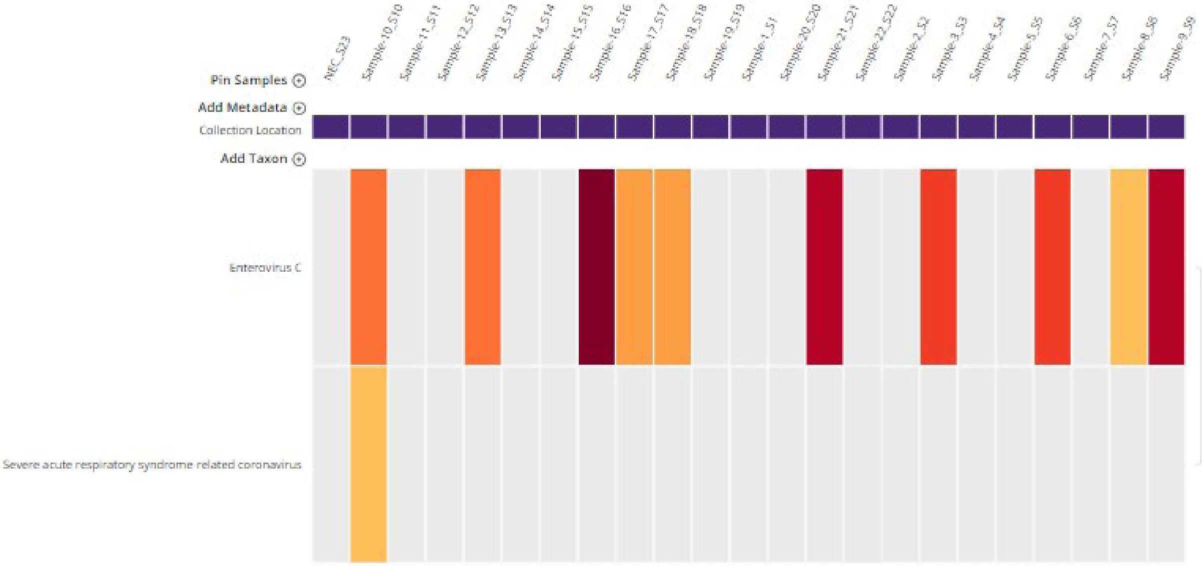
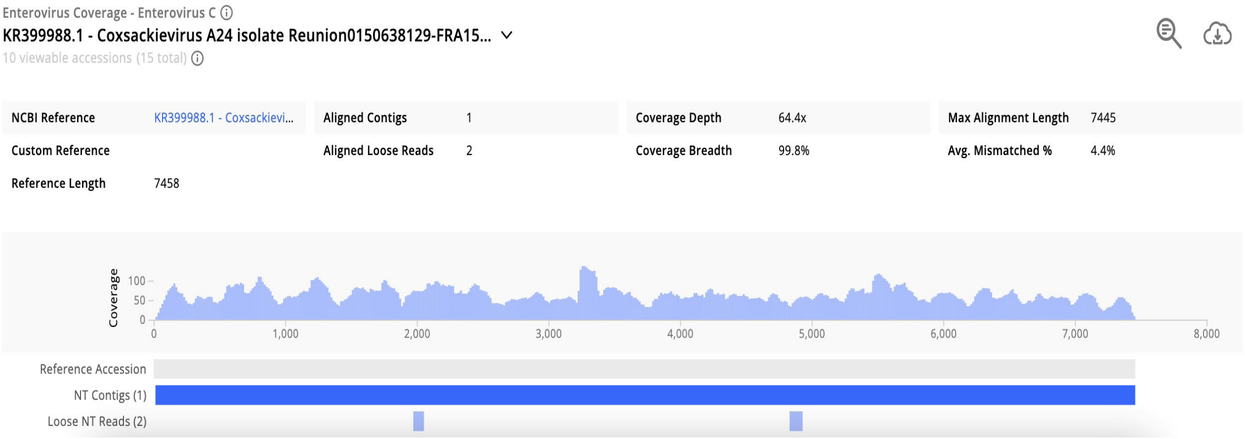


*Figure 2: **Heatmap of top hits, of viral origin, from sequencing of libraries for S1 to S22. This heatmap used the background model from eye samples of healthy controls with the threshold of NT rPM (nucleotide reads per million) >=10 and NT L (alignment length in basepairs: length of the aligned sequence)>=50.***



Enterovirus Coverage - Enterovirus C

KR399988.1 - Coxsackievirus A24 isolate Reunion0150638129-FRA15...

10 viewable accessions (15 total)

NCBI Reference	KR399988.1 - Coxsackievi...	Aligned Contigs	1	Coverage Depth	64.4x	Max Alignment Length	7445
Custom Reference		Aligned Loose Reads	2	Coverage Breadth	99.8%	Avg. Mismatched %	4.4%
Reference Length	7458						

Figure 3: Coverage visualisation of sample S16 for Enterovirus C.

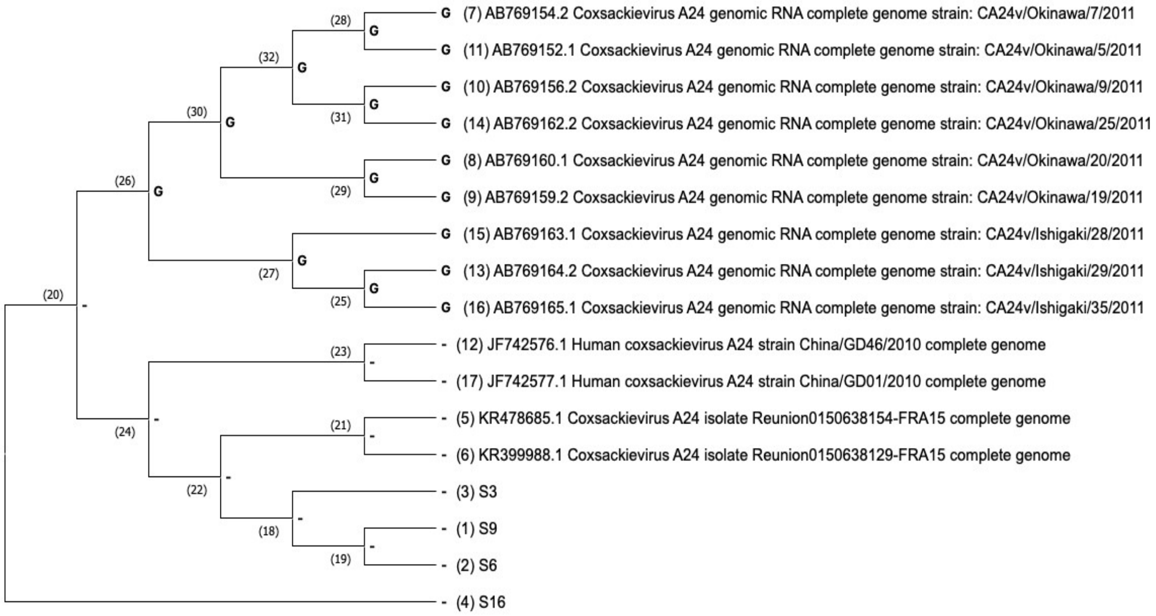


Figure 4: Phylogenetic analysis of four high coverage samples (S3, S6, S9, S16) against other coxsackievirus genomes, from NCBI, reported to have caused ocular infections.

DISCUSSION

The recent outbreak of conjunctivitis started in mid-July, peaked in August with half of the cases visiting the hospital, suffering from conjunctivitis.11 This prevalence then decreased in September 2023. The major pathogen causing the mass outbreak of conjunctivitis in 2023 was found to be Enterovirus C (coxsackievirus strain A24). Enterovirus family is one of the most common causes of viral conjunctivitis.12 However, this strain A24, first observed in 1970, is known to cause highly contagious conjunctivitis and has a short incubation period of 12 hours to 3 days.13,14 This might be the reason behind sudden spike of cases in Nepal as this same strain has previously been reported to cause several outbreaks throughout the world, with one study revealing import of the virus from Asia.15,16 Cutibacterium acnes, which is a normal flora and an opportunistic infectant was seen.17 The samples also had E. coli, which is known to cause eye infections such as conjunctivitis. Two samples (S11 and S16) had hits for Micrococcus luteus, known to cause keratitis, which needs to be further confirmed through polymerase chain reaction (PCR).18 Few samples also had Sphingomonas spp in them. Studies have shown that certain Sphingomonas spp. cause eye infections.19 Our findings correlated to results of PCR tests from

National Public Health Laboratory (NPHL), which found enterovirus in eight of ten samples collected from a hospital in Lalitpur, Nepal.20 However, the sub type and strain was not identified or reported in their analysis. The unbiased mNGS approach, in our investigation, was able to find numerous possible organisms, of viral or bacterial origin, in a single evaluation, including the strain. Thus, our investigation depicted the strain of enterovirus (A24) responsible for conjunctivitis outbreak in Nepal and supported the use of unbiased mNGS approach for deeper investigation of ocular infections, for instance in identification of viruses to strain level.

CONCLUSIONS

This study identified Enterovirus C (coxsackievirus A24) as the primary pathogen responsible for the 2023 conjunctivitis outbreak in Nepal. Further, the use of metagenomics next generation sequencing for strain level identification depicts the value of mNGS as an approach for comprehensive surveillance and investigations of infections, particularly during outbreak situations.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

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