









RESEARCH ARTICLE

REVISED Identification of coxsackievirus A24 variant during an acute hemorrhagic conjunctivitis outbreak in coastal Kenya, 2024

[version 2; peer review: 3 approved, 1 approved with reservations]

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<https://doi.org/10.12688/wellcomeopenres.23522.1>Latest published: 31 Mar 2025, 10:28
<https://doi.org/10.12688/wellcomeopenres.23522.2>**Abstract****Background**

In early 2024, a surge in acute hemorrhagic conjunctivitis (AHC), also referred as “red eye” disease, was observed in coastal Kenya, prompting the Ministry of Health to issue an outbreak alert. Herein, we investigated the etiology of this outbreak.


Methods

Ocular swabs were obtained from 13 individuals presenting with AHC at a Mombasa clinic in early February 2024. Ten of these were analyzed using bacterial cultures, and all 13 using a pan-adenovirus quantitative PCR (qPCR) and metagenomic sequencing. Potential viral

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etiology was confirmed by a specific qPCR, amplicon sequencing and phylogenetic analysis.

Results

Bacterial cultures yielded no growth except in three samples where non-pathogenic bacteria were detected. All 13 samples were adenovirus qPCR negative. Metagenomic sequencing detected coxsackievirus A24 variant (CA24v) in three of the 13 samples. CV-A24v detections were confirmed by both CV-A24v specific qPCR and amplicon sequencing of an approximately 450 nucleotide long VP4/2 junction genomic region. Phylogenetic analysis of the VP4/2 sequences showed that they were closely related to CV-A24v genotype IV.

Conclusion

The AHC epidemic in coastal Kenya in early 2024 was likely caused by CA24v. Metagenomic sequencing is a powerful tool for identifying potential causative agents of new disease outbreaks.


Plain language summary

In early 2024, there was a significant increase in cases of acute hemorrhagic conjunctivitis (AHC), commonly known as "red eye" or "pink eye" disease, in coastal Kenya, which led to the Ministry of Health issuing an outbreak alert. To understand the cause of this outbreak, we collected eye swabs from 13 patients at an eye clinic in Mombasa City, coastal Kenya. Initial testing of these samples was performed by checking for growth of pathogenic bacteria, and presence of a commonly known viral agent of AHC, adenovirus, using a polymerase chain reaction. The results showed no presence of harmful bacteria in the samples, and all tested negative for adenovirus. However, on further analysis of these samples by characterizing all non-human genetic material in the samples using an approach known as metagenomic sequencing, we detected the presence coxsackievirus A24 variant (CA24v) genetic material, a well-recognized cause of AHC, in three patients. The presence of this virus in the samples was confirmed through its specific in-house molecular test and a second genetic analysis approach. The CA24v strains detected in the samples were determined to be closely related to a known CA24v genotype assigned IV. Our findings suggest that the AHC disease outbreak observed in coastal Kenya in early 2024 was likely caused by CA24v. We highlight the usefulness of metagenomic sequencing as a tool for identifying the causes of new disease outbreaks, crucial for public health responses.

Keywords

Conjunctivitis, Red eye, Coxsackievirus A24 variant, Kenya, Africa, Enterovirus

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REVISED Amendments from Version 1

The changes done to this paper include:

1. An update to the abbreviation of the coxsackievirus A24 variant from CA24v to CV-A24v.
2. Spelling corrections for “conjunctivitis” to “conjunctivitis”.
3. An update in the methods section to include a detailed description of our:
 1. rotavirus group A screening method i.e., sample input, primers used and the thermocycling conditions.
 2. controls used in the adenovirus screenign method.

bacteria identification method using MALDI-TOF.

Any further responses from the reviewers can be found at the end of the article

Background

Acute haemorrhagic conjunctivitis (AHC) is a highly contagious eye disease first reported in 1969 in Ghana¹. The syndrome, also referred to as “red eye”, “pink eye” or “Apollo 11 disease”, is characterised by one or more symptoms that include sudden onset ocular pain, foreign body sensation, sub-conjunctival haemorrhage, itching, photophobia, swollen eyelids, eye discharge, epiphora and blurred vision². Systemic symptoms like fever, runny nose, congestion, limb pain, fatigue, and preauricular lymphadenopathy may also appear in some cases. Several AHC outbreaks have been reported in tropical and subtropical countries³.

AHC disease is often self-limiting but results in individuals missing school or work, leading to economic losses⁴, and school closure and/or case exclusion is a common strategy to curtail AHC transmission. During outbreaks, increased ophthalmologist clinic visits occur, and antibiotic prescription is common^{5,6}. The latter can indirectly contribute to the development of antibiotic resistance as most epidemics are in fact caused by viruses^{7,8}. Severe complications following infection are rare except for limited cases of radiculomyelitis (inflammation of the spinal root nerves and cord in ~1 in 10,000 infections)^{5,9}. Mismanagement including self-medication and visiting traditional healers can exacerbate the situation^{10,11}.

AHC can result from viral, bacterial or allergen exposure^{12–14}. Viral causes are characterised by explosive outbreaks, with a short incubation time (12–48 hrs) and rapid spread^{15–17}. Three major viruses are associated with AHC; specific adenovirus types, enterovirus 70, and the coxsackievirus A24 variant (CA24v)⁵. Other viruses such as SARS-CoV-2, measles, herpes simplex virus and varicella zoster virus have also been implicated in AHC clinical cases^{14,18}. Adenovirus AHC is mostly accompanied with keratitis^{10,19}. Laboratory diagnosis is therefore key to confirming AHC aetiology²⁰.

Following the COVID-19 pandemic, AHC outbreaks have been reported in Asia (India, Pakistan, Nepal) since 2022^{18,21–24}

and more recently in multiple countries in East and Southern Africa. In January 2024, the Kenya Ministry of Health issued an outbreak alert over increased cases viral conjunctivitis on the Kenyan Coast. The disease was reported to have spread extensively especially in Mombasa and Kilifi counties, with sporadic cases observed elsewhere in the country. We present the first investigation in Africa identifying CV-A24v as a potential etiological agent of 2024 AHC outbreaks in the region.

Methods

Ethics consideration

The samples analysed here were collected as part of Ministry of Health outbreak response activities to the AHC outbreak. In such scenarios in the country, informed consent (verbal or written) is not considered an essential step prior specimen collection. Reporting of molecular diagnostics and sequencing analysis in scenarios of outbreak response by Kenya Medical Research Institute (KEMRI) - Wellcome Trust Research Programme (KWTRP) was approved by KEMRI Scientific Ethics Review Unit (SERU) Committee Institutional Review Board (IRB) based in Nairobi, Kenya on May 19th, 2024 (Protocol #: **KEMRI/SERU/CGMR-C/304/4894**).

Study population

On February 6th 2024, 13 patients attending an outpatient eye clinic with unilateral or bilateral AHC, in Mombasa, provided an eye swab. No additional demographic details about the patients were shared. Mombasa city is Kenya’s second largest city, has a population of ~1.2 million people, is located on the Kenyan coast and has a tropical climate. The samples were collected using a sterile general-purpose swab and immediately placed into universal transport media (UTM) and then transported to KWTRP, Kilifi, Kenya, at ambient temperature.

Bacterial culture

To observe bacterial growth, the swabs from 10 samples were inoculated in blood agar, chocolate agar and MacConkey agar then incubated at 37°C in 5% CO₂ for 18 hours. The remaining three lacked labels and were excluded from bacterial culture processing. To identify the bacteria we used Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF).

Pan-adenovirus screening

Nucleic acids including DNA were extracted from 140µl of the 13 ocular swabs UTM by QIAamp Viral Mini-RNA kit (QIAGEN, UK) following the manufacturer’s instructions. These were then screened using a pan-adenovirus real-time PCR assay targeting the hexon gene of adenoviruses²⁵. The following primer sequences were used: forward primer (5’ - GCCCAGT-GGTCTTACATGCACATC - 3’), probe (FAM -TCGGAGTAC-CTGAGCCCCGGGTCTGGTGCA - MGBNFBQ), and reverse primer (5’ - GCCACGGTGGGGTTTCTAAACTT - 3’). The thermocycling conditions were set up as follows: 95°C for 20 seconds and 40 cycles of 94°C for 15 seconds and 60°C for 30 seconds. An adenovirus positive and negative control and a non-template control were included in the assay.

Metagenomic screening

The nucleic acid extracts extracted above (n=13) were treated with TURBO™ DNase (Invitrogen, Carlsbad, CA) to deplete any DNA in the sample. First strand synthesis was performed using Superscript IV first strand synthesis kit (ThermoFisher Scientific, UK) and FR26RV-ENDOH primers as per the manufacturer's instructions²⁶. Second strand synthesis was done using Klenow fragment 3' to 5' exo- (New England BioLabs) by incubating for 30 minutes at 37°C. The cDNA was then amplified using Q5® Hot Start High-Fidelity 2X Master Mix (New England BioLabs, USA) and FR20RV primer (5'-GCCGGAGCTCTGCAGATATC-3'). The thermocycling conditions were set up as follows: 98°C for 30 seconds, 40 cycles of 98°C for 10 seconds, 54°C for 30 seconds and 72°C for 30 seconds and a final extension of 72°C for 2 minutes. The PCR product was used to prepare libraries using the SQK-LSK114 ligation kit with SQK-NBD114.96 native barcoding Kit (Oxford Nanopore Technologies, UK) and sequenced on the GridION Flow Cell (R10.4.1) (Oxford Nanopore Technologies, UK).

Confirmation of detection of CV-A24v and VP4/VP2 sequencing

CV-A24v is a positive-sense RNA virus from the genus *Enterovirus*, family *Picornaviridae*. The single-stranded CV-A24v genome is approximately 7400 bp in length and encodes 4 capsid proteins (VP4, VP2, VP3, and VP1) and 7 non-structural proteins (2A-2C, and 3A-3D). Molecular epidemiological studies of CV-A24v often utilize the VP1 (receptor binding) or 3C (viral protease) regions of the CV-A24v genome. Currently, eight genotypes of CV-A24v are described (I–VIII) and responsible for major global epidemics of AHC²⁷.

To confirm the presence CA24v, all 13 samples were reanalysed using a CV-A24v specific quantitative PCR targeting the 5' untranslated region (UTR) genomic region. Primers/probe sequences from a previous study were used²⁸. The sequences were as follows: Forward primer: 5'-CCAACCACGGAGCAGGTGA-3'. Reverse primer: 5'-GAAACACGGACACCCAAAGTAGT-3'. Probe: 5' FAM-CAACCCAGCAACTAGCCTGTCTGTAACCG-MGBNFQ 3'. Additionally, the nucleic acid extracts from the positive samples were amplified using VP4/VP2 primers (Forward primer: CCGGCCCTGAATGYGGCTAA and Reverse primer: TCWGGHARYTTCCAMCACCANCC) as previously described²⁹. Library preparation was performed using the Illumina COVIDSEQ kit, (Illumina, California, USA) as per the manufacturer's protocol, and the libraries were then sequenced on the Illumina Miseq platform.

Rotavirus group A screening

Nucleic acids extracted as described above were used to screen for RVA using a realtime PCR assay targetting the NSP3 gene. The primers and probe were adopted from elsewhere: Forward primer (5'-'ACCATCTWCACRTRACCCTCTATGAG-3'), Probe (VIC-AGTAAAAGCTAACACTGTCAAA 32-NFQMGB) and reverse primer (GGTCACATAACGCCCTATAGC)¹. The thermocycling conditions were as follows: 95°C for 20 seconds and 40 cycles of 94°C for 15 seconds and 60°C for 30 seconds.

Data analysis

Metagenomic data. The barcodes and adapters were trimmed from the sequence data using porechop v.0.2.4 (<https://github.com/rwick/Porechop>). Taxonomic classification was then performed on the trimmed data to identify potential pathogens in the samples using the Nanopore mNGS Pipeline v.0.7 available on the Chan Zuckerberg ID webserver (i.e., CZ ID)³⁰.

Amplicon sequence data. The fastq data was checked for quality using FastQC v0.12.1 (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>), cleaned using FastP v0.23.4 (<https://github.com/OpenGene/fastp>), mapped to the reference (Genbank accession PP548237) and the consensus genome generated using ivar v.1.3.1³¹.

Phylogenetic analysis. The VP4/VP2 segments of CV-A24v obtained through amplicon sequencing, as well as VP3 segments obtained from metagenomic sequencing, were used to estimate maximum likelihood trees. Global CV-A24v genomes were downloaded from GenBank on 05/07/2024. The recovered partial sequences were aligned using MAFFT v7.487 (<https://mafft.cbrc.jp/>), with the global CV-A24v sequences, trimmed to partial VP3 for the metagenomic data and VP4/VP2 segments for the amplicon sequencing data. Maximum likelihood trees were then estimated using the IQ-Tree2 v 2.1.3 software (<http://www.iqtree.org/>), employing the best-fit model of nucleotide substitution identified using the lowest Bayesian Information Criterion (BIC) and 1000 bootstrap replicates. Representative sequences from the eight known CV-A24v genotypes²⁷ were included on the phylogeny to identify the genotype present in the newly obtained Kenya CV-A24v sequences.

Results

Bacterial culture and adenovirus screening

No growth was observed in seven of the 10 processed samples through bacterial culture. Three cultures established non-pathogenic bacterial growth (*Actinocorallia libanotica*, *Lactobacillus plantarum*, and *Staphylococcus epidermidis*). Adenovirus quantitative PCR screening was negative for all 13 samples processed.

Metagenomic sequencing and genotyping

All 13 samples processed by metagenomic sequencing. A total of 5.94 million reads were recovered, although most (~90%) mapped to the human genome or non-pathogenic organisms (Table 1). None of the bacteria detected were likely pathogens. However, interestingly, in three samples, metagenomic sequence reads that mapped to CV-A24v were detected (Table 1). In addition, three samples contained genetic sequence reads that mapped to rotavirus group A (RVA), and one had sequence reads that mapped to both CV-A24v and RVA. All the 13 samples were reanalysed using a CV-A24v and RVA specific quantitative PCR³². Whereas all the samples gave a RVA negative PCR, the three samples with CV-A24v reads amplified with a cycle threshold values of 32.29, 38.43 and 37.65.

To characterize the strain of CV-A24v in the three coastal Kenya samples, we amplified and sequenced the VP4/2

Table 1. Results of the metagenomic analysis. A total of 13 clinical samples were processed together with an NC-Extraction, NTC-PCR, NC-library prep. Samples that contain CV-A24v are shaded grey.

Sample	Number of reads	Major virus (Aligned reads)	Major bacteria (Aligned reads)	BLAST results
1	235,256	<i>Orthopneumovirus</i> (2)	<i>Cupriavidus</i> (578), <i>Pseudomonas</i> (150), <i>Acinetobacter</i> (52)	
2*	367,790	<i>Orthopneumovirus</i> (1)	<i>Cupriavidus</i> (566), <i>Pseudomonas</i> (349)	
3	116,714	<i>Rotavirus</i> (1)	<i>Cupriavidus</i> (279), <i>Pseudomonas</i> (163), <i>Bacillus</i> (75)	BLAST hits to Rotavirus
4	321,821	<i>Rotavirus</i> (20), <i>Orthonairovirus</i> (4)	<i>Cupriavidus</i> (1519), <i>Pseudomonas</i> (1085), <i>Sphingobium</i> (158)	Blast hits to Rotavirus A
5	204,747	<i>Orthonairovirus</i> (4), <i>Mammarenavirus</i> (1)	<i>Cupriavidus</i> (1458), <i>Pseudomonas</i> (661), <i>Acinetobacter</i> (395)	
6	228,966	<i>Orthonairovirus</i> (3), <i>Mammarenavirus</i> (2), <i>Betapapillomavirus</i> (2)	<i>Cupriavidus</i> (2389), <i>Pseudomonas</i> (981), <i>Cutibacterium</i> (713)	
7	216,156	None	<i>Cupriavidus</i> (469), <i>Pseudomonas</i> (137), <i>Staphylococcus</i> (91)	
8	355,222	<i>Orthobunyavirus</i> (1)	<i>Staphylococcus</i> (913), <i>Cupriavidus</i> (86), <i>Escherichia</i> (11)	
9	88,755	None	<i>Cupriavidus</i> (2677), <i>Pseudomonas</i> (2603), <i>Acinetobacter</i> (441)	
10	292,219	<i>Enterovirus</i> (6), <i>Flavivirus</i> (14), <i>Orthonairovirus</i> (1)	<i>Cupriavidus</i> (52), <i>Staphylococcus</i> (89), <i>Escherichia</i> (25)	Blast hits to Coxsackievirus A24
11	192,360	<i>Enterovirus</i> (4), <i>Orthonairovirus</i> (1), <i>Orthobunyavirus</i>	<i>Cupriavidus</i> (43), <i>Staphylococcus</i> (43), <i>Escherichia</i> (27)	Blast hits to Coxsackievirus A24
12	92,964	None	<i>Cupriavidus</i> (36), <i>Staphylococcus</i> (27), <i>Bacillus</i> (17)	
13	256,147	<i>Enterovirus</i> (8), <i>Rotavirus</i> (1)	<i>Cupriavidus</i> (51), <i>Staphylococcus</i> (47), <i>Escherichia</i> (23)	Blast hits to Coxsackievirus A24 and Rotavirus A
NC-Extraction	3,282	None	<i>Pseudomonas</i> (58), <i>Cutibacterium</i> (40), <i>Bifidobacterium</i> (27)	
NTC-PCR	391	None	<i>Bifidobacterium</i> (43), <i>Limosilactobacillus</i> (23)	
Negative control	355	None	None	

junction region and undertook phylogenetic analysis including known genotype representative sequences. The alignment of the three Kenya CV-A24v VP4/2 sequences showed presence of 3 nucleotide changes. Phylogenetic analysis showed that the sequences documented here clustered closely with the GIV genotype (Figure 1). Further, the coastal Kenya sequences showed a close relationship with sequences from Mayotte (Indian Ocean Island) that were sampled in 2024. Based on the VP1 sequences, the Mayotte sequences cluster within the cluster IV of the GIV genotype like Kenyan sequences sampled in 2010.

Discussion

Like in many parts of the world, the causes of AHC epidemics are not typically investigated in Kenya. This can be attributed

to the limited diagnostic capacities in most outbreak settings and the self-limiting nature of the syndrome. However, several potential pathogens can be responsible, making it important to understand aetiology for appropriate public health advice. In the past, adenoviruses were mostly assumed to be the cause of AHC outbreaks, but this was rarely confirmed.

On 22nd January 2024, the Kenya Ministry of Health issued an alert of an epidemic of AHC with the coastal region recording most cases³³. We set out to determine disease etiology by processing a small set of samples using a pan-adenovirus quantitative PCR analysis and bacterial culture on ocular swabs from symptomatic cases. These returned negative results. However, by using metagenomic sequencing we detected

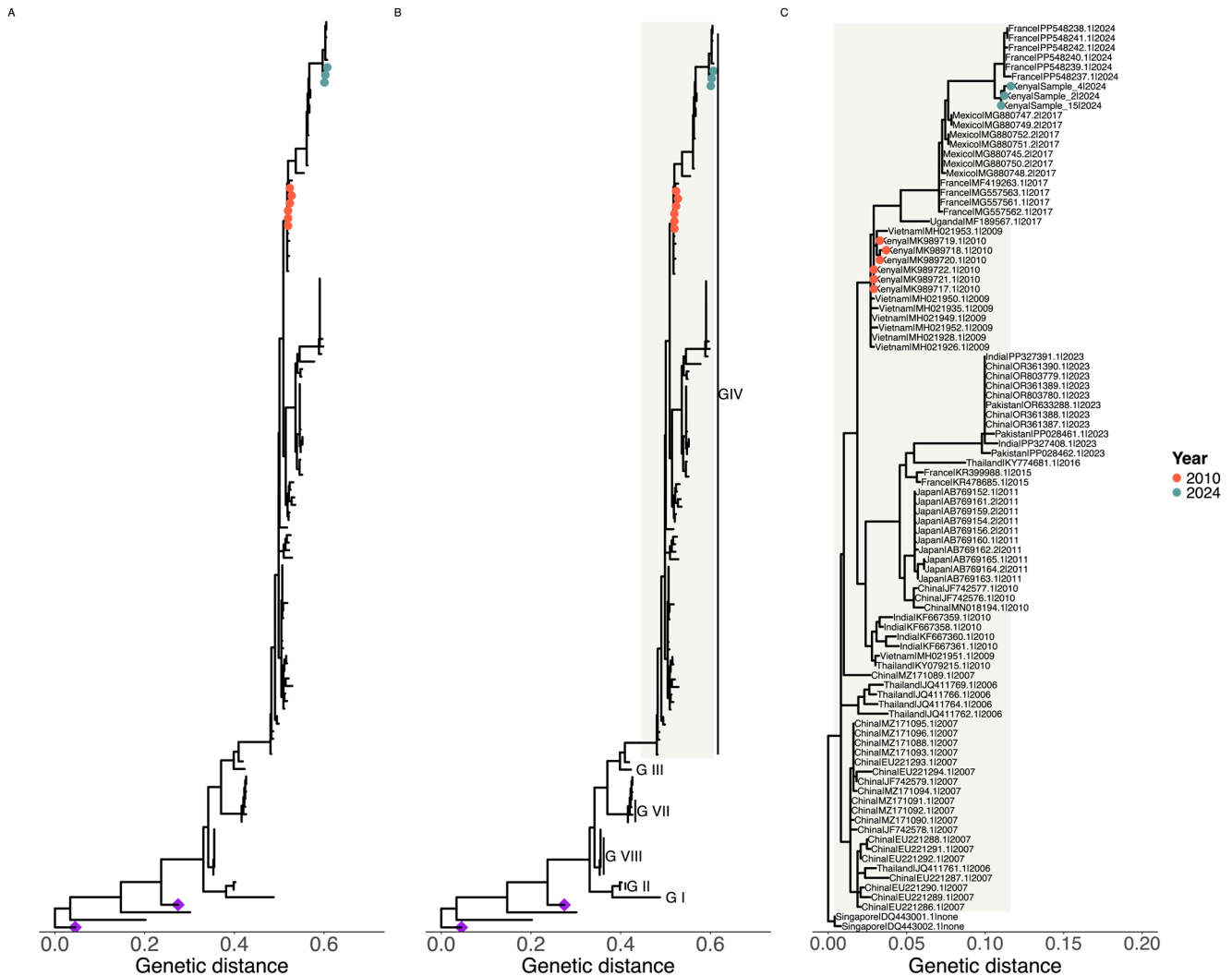


Figure 1. Phylogenetic placement of coxsackievirus A24 variant (CA24v) sequences from Kenya in a global context. Panel A shows the phylogeny based on VP3 sequences (n=132) including the Kenyan CV-A24v sequences recovered from metagenomic sequencing. Panel B shows the phylogeny based on VP4/VP2 sequences (n=158) including the Kenya data recovered by amplicon sequencing. Panel C shows a detailed phylogeny of genotype IV, showing the Kenyan sequences within the global context. The colored tip-points show Kenyan sequences from 2010 (orange) and 2024 (green). The purple diamonds show environmental CV-A24v sequences that clustered differently from the rest of the sequences.

CV-A24v sequences in three cases that were then confirmed by qPCR.

CV-A24v was first isolated in Singapore in 1970³⁴, and has been recognized as one of the leading causes of AHC epidemics worldwide, typically occurring in densely populated tropical and subtropical areas. Few outbreaks of CV-A24v have occurred in the western hemisphere (mostly localised^{35,36}), likely due to better hygiene standards, limited overcrowding, and unfavourable climate conditions. Since 2022, there have been several reports of CV-A24v AHC outbreaks in parts of Asia and Africa^{37–41}.

To date, there have been few phylogenetic studies of CVA24v-related AHC outbreaks in the Africa⁴¹. Our metagenomic

sequencing recovered part of the VP3 gene (135 bases) while amplicon sequencing generated parts of VP4/VP2 (450 bases), regions of the CV-A24v genome that are rarely used in CV-A24v genomic epidemiology. Phylogenetic analysis of these sequences in the global contextual data indicated the circulating CA24 strains in Kenya fell within genotype IV (GIV) that has been the dominant genotype in many recent outbreaks globally⁴¹.

This is the first report of confirmed detection of CV-A24v in cases of AHC in the Kenyan population. We previously detected CV-A24v during a household-based respiratory syncytial virus (RSV) surveillance study in Kilifi, coastal Kenya, in 2010⁴². Six cases (aged between 8.5–33 months) were identified from 95 metagenomically sequenced respiratory samples.

The cases originated from six different households, none presented with conjunctivitis, one had diarrhoea, but all had rhinorrhoea. Based on VP1 sequences, the 2010 detections formed a monophyletic group within genotype GIV, but separate to that observed here for the 2024 outbreak.

The CV-A24v outbreak reported here is one of the multiple AHC outbreaks recorded in East Africa over the last two decades. CV-A24v was confirmed to be responsible for an AHC outbreak in June 2010 and July 2010, in Uganda (>26 districts, >6000 cases) and Southern Sudan (>400 cases), respectively⁴³. Between November 2016 and January 2017, a CV-A24v AHC outbreak was observed in a prison, remand and police station in Gulu district, Uganda⁴⁴. In 2024, AHC outbreaks have been reported in several countries in Eastern and Southern Africa including Tanzania⁴⁵, Uganda⁴⁶, Burundi⁴⁷, Malawi⁴⁸, Zambia⁴⁹, South Sudan⁵⁰ and South Africa⁵¹. In Kenya the epidemic seems to have spread beyond the coastal region to western region (Busia, Kisii, Siaya) and Nairobi City.

Our analysis here was limited by the small number of samples that were all collected from one geographic region in the country with no additional metadata. In addition, our metagenomic data comprised six million reads from a single GridION flow cell, predominantly mapping to the human genome, with few aligning to viruses as expected. Viral detections such as rotavirus that were not confirmed by targeted qPCR were ignored henceforth. The non-detection of CV-A24v in some samples may be attributed to (a) low sample quality (at collection, transport, storage), (b) timing of sample collection (i.e., the different stages of illness), and (c) the low sensitivity of metagenomic sequencing at this depth. Further, we cannot rule out that some of the AHC cases were a result of other causes than CA24v. As our metagenomic and amplicon sequencing recovered portions of CV-A24v genome that are not commonly used in genomic epidemiology, we are now establishing a whole genome sequencing platform to conduct further work on the virus.

This is the first study in Africa to identify CV-A24v as a possible cause of the recent AHC outbreaks in the region. Our findings were promptly shared with the Ministry of Health to assist with a public health response, and by collaborating with the Ministry we were able to determine the most likely cause of the AHC epidemic. Our study also demonstrates the power of metagenomic sequencing in identifying etiological agents in new epidemics. Despite this, several questions remain unanswered concerning CV-A24v epidemiological patterns and strain dynamics in the local population and the broader region. Detailed longitudinal investigations are needed to address these questions.

Ethics and consent

The samples analysed here were collected as part of Ministry of Health outbreak response activities to the AHC outbreak and as such written informed consent is not considered an essential step prior specimen collection. In such cases, individual

consent is typically not required. Our analysis presented data that has been adequately anonymized as approved by the Institutional Review Board (IRB), allowing us to publish the outcomes of the outbreak investigations. Molecular diagnostics and sequencing analysis in scenarios of outbreak response by Kenya Medical Research Institute (KEMRI) - Wellcome Trust Research Programme (KWTRP) was approved by KEMRI Scientific Ethics Review Unit (SERU) Committee based in Nairobi, Kenya on May 19th, 2024 (Protocol #: **KEMRI/SERU/CGMR-C/304/4894**).

Data availability

Underlying data

The VP4/2 sequences have been deposited into GenBank under accession numbers (PQ034605, PQ034606 and PQ034607).

GenBank: Genomic data for Coxsackievirus A24 isolate KEN/MSA/15/2024 partial polyprotein gene. Accession number PQ034605; <https://www.ncbi.nlm.nih.gov/nucleotide/PQ034605>⁵².

Identifier: <http://identifiers.org/ncbiprotein:XCX13095.1>⁵³

GenBank: Genomic data for Coxsackievirus A24 isolate KEN/MSA/2/2024 partial polyprotein gene. Accession number PQ034606; <https://www.ncbi.nlm.nih.gov/nucleotide/PQ034606>⁵⁴.

Identifier: <http://identifiers.org/ncbiprotein:XCX13096.1>⁵⁵

GenBank: Genomic data for Coxsackievirus A24 isolate KEN/MSA/4/2024 partial polyprotein gene. Accession number PQ034607; <https://www.ncbi.nlm.nih.gov/nucleotide/PQ034607>⁵⁶.

Identifier: <http://identifiers.org/ncbiprotein:XCX13097.1>⁵⁷

Authors contributions

LIO-O, GG, CAN conceptualised the study

FK, MM, NM, PM oversaw sample collection

SM did the bacterial cultures

EM, JM, AWL did the metagenomic sequencing and phylogenetic analysis

AWL, JMM & CNA wrote the first draft of the manuscript.

ECH, CNA, LIO-O, GG, JM, PA, EB, CH, and NM, revised the manuscript.

LIO-O, GG, CNA provided grant funding and overall project supervision

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Version 2

Reviewer Report 07 May 2025

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 **Savitri Sharma** 

Jhaveri Microbiology Center, Brien Holden Eye Research Centre (BHERC), L V Prasad Eye Institute (LVPEI), Hyderabad, Telangana, India

The response to comments is satisfactory

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: clinical ocular microbiology. Research in fungal keratitis, viral keratitis, microsporidial keratitis, endophthalmitis, Acanthamoeba keratitis etc.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 23 April 2025

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 **Sanjay Tikute**

ICMR- National Institute of Virology, Pune, India

1. The revised manuscript is checked for the clarifications to the comments raised. Most of them can be accepted. Still in conclusion, CV24v is typed in wrong manner. Please change it as CV-A24v.
2. In plain language summary, the same mistake is repeated mentioned in point no.1. The manuscript can be accepted ONLY after the above corrections are done.
Thanks...

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Virus isolations, Infectious diseases diagnosis, Viral pathogenesis, Laboratory animal experimentation.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 11 April 2025

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Anchal Thakur

Post Graduate Institute of Medical Education and Research, Chandigarh, India

No further comments

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Conjunctivitis, Cataract, Iol, quality of vision

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 26 February 2025

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Savitri Sharma 

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(LVPEI), Hyderabad, Telangana, India

This study identified the cause of acute hemorrhagic conjunctivitis in 13 patients using meta genomic platform. Bacterial culture and real time PCR for adenovirus were also done. The methodology is described well for most part except for information on sample collection.

1. Please clarify whether different swabs were collected for different tests. It would be good to describe the sample collection method for each of the tests.

2. **Methods-Bacterial culture:** The method adopted is inadequate in terms of incubation time which should be at least 48 hours. The method of identification of the bacteria has not been given. This lacunae may be noted as a limitation.

3. Clinical data is completely missing. The number being small it should have been possible to describe the clinical features along with representative figures. Stage of the disease might have been variable in the patients that may explain why all cases were not positive for the virus.

The manuscript is well written.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

No

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: clinical ocular microbiology. Research in fungal keratitis, viral keratitis, microsporidial keratitis, endophthalmitis, Acanthamoeba keratitis etc.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 21 Mar 2025

Arnold Wasike Lambisia

1. Please clarify whether different swabs were collected for different tests. It would be good to describe the sample collection method for each of the tests.

Our response: Thank you for the comment. We clarify that only ocular swabs were collected and these were used for all the laboratory analysis described; for cultures and nucleic acid analysis approaches that were used for the different molecular tests in the article.

2. **Methods-Bacterial culture:** The method adopted is inadequate in terms of incubation time which should be at least 48 hours. The method of identification of the bacteria has not been given. This lacunae may be noted as a limitation.

Our response: An incubation time of 18-24 hours was sufficient to identify the suspected pathogens and other bacteria known to cause "red eye" e.g., Staphylococcus and Streptococcus. The method of identification the bacteria is added in the methods.

3. Clinical data is completely missing. The number being small it should have been possible to describe the clinical features along with representative figures. Stage of the disease might have been variable in the patients that may explain why all cases were not positive for the virus.

Our response: These were outbreak samples collected at one timepoint i.e when the individuals visited the clinic with "red eye" as the symptom no further clinica data was provided to describe what stage of the infection the patients were in. We appreciate that this wa one of the limitations of the article and we follow up work with larger sample size underway.

The manuscript is well written. Our response: Thank you for a positive view of our work.

Competing Interests: No competing interests were disclosed.

Reviewer Report 24 February 2025

<https://doi.org/10.21956/wellcomeopenres.25943.r118289>

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**Sanjay Tikute**

¹ ICMR- National Institute of Virology, Pune, India

² ICMR- National Institute of Virology, Pune, India

Title: Identification of coxsackievirus A24 variant during an acute hemorrhagic conjunctivitis outbreak in coastal Kenya, 2024.

General Comments: (Need to revise the manuscript before indexing).

1. In abstract (result section) coxsackievirus A24 is written in short form as 'CV24v'. Please change it and rewrite as "CV-A24v". Also correct it in entire article.
2. The outbreak was in the coastal area of Kenya. Therefore more population should be considered for the study. Thirteen patients is a very low number of study subjects.
3. Clinical details or symptomatic conditions of all the patients should be included.
4. In background after the word blurred vision the sign '=' is added, please remove it.
5. In study population paragraph, it is mentioned that after sample collection it was transported to KWTRP at ambient temperature which is not advisable.
6. Metagenomic screening paragraph, the word 'incubating' is written as 'Incubating' remove capital and small one.
7. In abstract, in the result section, it is mentioned that VP4/2 junction, please explain.
8. In metagenomics sequencing paragraph, it is mentioned that 3 samples mapped to RVA. After reanalyzing with specific primers all the samples were RVA negative. Add about the primers used and explain about negative results.
9. The Ct values of the 3 samples ranged between 32.29 to 37.65, what was the positive control and its value.
10. In table number 1, it is shown that only 2 samples that is , sample 3 and sample 4 after blast hits to rotavirus. What about the third sample previously mentioned. Needs to specify.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Partly

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

I cannot comment. A qualified statistician is required.

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Virus isolations, Infectious diseases diagnosis, Viral pathogenesis, Laboratory animal experimentation.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 21 Mar 2025

Arnold Wasike Lambisia

1. In abstract (result section) coxsackievirus A24 is written in short form as 'CV24v'. Please change it and rewrite as "CV-A24v". Also correct it in entire article. Our response: Thanks – This is now corrected

2. The outbreak was in the coastal area of Kenya. Therefore more population should be considered for the study. Thirteen patients is a very low number of study subjects. Our response: The samples were obtained as part of an outbreak response initiative by the ministry of health. This was not a research study. In such scenarios a few purposively selected samples can be adequate to tease out the cause of the outbreak disease. This convenience sampling approach is a limitation of the analysis we present. Indeed we have currently a larger molecular epidemiological study underway that will reinforce our findings here.

3. Clinical details or symptomatic conditions of all the patients should be included. Our response: Unfortunately more detailed symptom data of the cases we analysed are not available to us. As previously indicated, the samples were collected for outbreak response to determine the aetiology of AHC epidemic. The only symptom recorded from the patients was having a "red eye"/ AHC.

4. In background after the word blurred vision the sign '=' is added, please remove it. Our response: This has been removed

5. In study population paragraph, it is mentioned that after sample collection it was transported to KWTRP at ambient temperature which is not advisable. Our response: We agreed that this is not advisable. This may have affected the integrity of the samples and moving forward we stress cold chain transport.

6. Metagenomic screening paragraph, the word 'incubating' is written as 'Incubating' remove capital and small one. Our response: This has been corrected

7. In abstract, in the result section, it is mentioned that VP4/2 junction, please explain. Our response: The polyprotein has multiple gens which include the VP4 and VP2 so the primers target the VP4/VP2 junction.

8. In metagenomics sequencing paragraph, it is mentioned that 3 samples mapped to RVA. After reanalyzing with specific primers all the samples were RVA negative. Add about the primers used and explain about negative results. Our response: The primers have been added to the methods section. Since the rotavirus reads in the metagenomic sequence output were very few and this was not confirmed on PCR processing. We assume this may have been contaminants in the samples perhaps in the sample processing steps.

9. The Ct values of the 3 samples ranged between 32.29 to 37.65, what was the positive control and its value. Our response: We did not have a positive control for CV-A24v at the time of running the assay. This was not a commercial kit.

10. In table number 1, it is shown that only 2 samples that is , sample 3 and sample 4 after blast hits to rotavirus. What about the third sample previously mentioned. Needs to specify. Our response: That was sample 13 shown in table 1

Competing Interests: No competing interests were disclosed.

Reviewer Report 24 February 2025

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Milton Mogotsi

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Review Report:

Title: Identification of coxsackievirus A24 variant during an acute hemorrhagic conjunctivitis outbreak in coastal Kenya, 2024.

The study by Lambisia and colleagues investigated the aetiology of an acute haemorrhagic conjunctivitis (AHC) outbreak in Kenya's coastal region in 2024. Utilizing metagenomic sequencing, the authors identified Coxsackievirus A24 variant (CA24v) as the potential causative agent, marking the first confirmed detection of CA24v in Kenyan AHC cases. Phylogenetic analysis placed the Kenyan strains within genotype IV (GIV), consistent with recent global outbreaks. The findings underscore the utility of metagenomic sequencing in identifying pathogens in new epidemics and highlight the need for enhanced diagnostic capacities in outbreak settings.

Comments:

The study addresses a significant public health concern by identifying the potential causative agent of a recent AHC outbreak in Kenya, providing critical information for public health responses. Metagenomic sequencing allowed for the detection of CA24v, a pathogen not typically identified through conventional diagnostic methods. On the other hand, phylogenetic analysis offered valuable insights into the genetic relationship of the Kenyan strains with global CA24v variants, contributing to the understanding of the virus's epidemiology. However, the study's conclusions are based on a small number of samples from a single geographic region, which may not fully represent the broader epidemiological landscape of AHC in Kenya. Additionally, the absence of detailed patient demographics and clinical data limits the ability to assess the full impact of the outbreak and the virus's behaviour in different populations. Furthermore, the study relies on samples from symptomatic cases, which may not capture the full spectrum of infection, including asymptomatic or mild cases.

Moreover, I have a concern regarding the absence of a positive control especially in the real-time PCR assay for adenovirus detection. We cannot conclude with certainty that the samples were negative for adenovirus when there was no positive control included. The negative results could be a results of PCR conditions, or issues with the primers, for instance. Also, the authors performed DNase treatment to digest all DNA in the samples prior cDNA and metagenomic sequencing. However, I am of the opinion that the authors should have processed the samples in parallel (DNase-treated and DNase-non treated) to confirm if indeed the samples were adenovirus negative considering that it is a DNA virus and it might have been removed by nuclease digestion.

My recommendations regarding this paper are that future studies should include a larger and more diverse sample set from multiple regions to provide a more comprehensive understanding of the outbreak's scope and the virus's epidemiology. Authors need to also incorporate detailed patient demographics, and clinical presentations which will be useful in assessing the impact of the outbreak and inform targeted public health interventions.

I concur with the authors that long-term surveillance and studies are necessary to monitor the persistence and evolution of CA24v in the region, as well as to evaluate the effectiveness of public health measures implemented in response to the outbreak. While the findings are significant, providing valuable insights into the aetiology of the AHC outbreak in Kenya, I strongly recommend that the authors consider publishing these results as a brief report instead of a full research article due to the above-mentioned limitations such as small sample size and lack of demographic/clinical data.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Partly

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Not applicable

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Virology, metagenomics, infectious disease

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 21 Mar 2025

Arnold Wasike Lambisia

Comments:

The study addresses a significant public health concern by identifying the potential causative agent of a recent AHC outbreak in Kenya, providing critical information for public health responses. Metagenomic sequencing allowed for the detection of CA24v, a pathogen not typically identified through conventional diagnostic methods. On the other hand, phylogenetic analysis offered valuable insights into the genetic relationship of the Kenyan strains with global CA24v variants, contributing to the understanding of the virus's epidemiology. **However, the study's conclusions are based on a small number of samples from a single geographic region, which may not fully represent the broader epidemiological landscape of AHC in Kenya.** Additionally, the **absence of detailed patient demographics and clinical data limits** the ability to assess the full impact of the outbreak and the virus's behaviour in different populations. Furthermore, the study relies on samples from symptomatic cases, which may not capture the full spectrum of infection, including asymptomatic or mild cases. Our response: Thanks for these comments. We agree that our study is indeed limited by the small number samples analysed coupled to

lack of detailed clinical data. This to some extent limits our conclusions. However we do still think this analysis gives us some important first insight on the likely cause of the AHC outbreak we recorded. Follow up bigger and carefully designed epidemiological study analysing samples collected over a broader geographical area and from individuals with and without these symptoms or of different clinical severity will be an important follow up to our findings here. Indeed we are already currently leveraging a study that was going on in the community at this time of the outbreak that collected serial samples and detailed clinical data from the same individual and ongoing analysis will identify the role of the virus in symptomatic and asymptomatic individuals and other potential pathogens involved in AHC.

Moreover, I have a concern regarding the absence of a positive control especially in the real-time PCR assay for adenovirus detection. We cannot conclude with certainty that the samples were negative for adenovirus when there was no positive control included. The negative results could be a result of PCR conditions, or issues with the primers, for instance. Also, the authors performed DNase treatment to digest all DNA in the samples prior to cDNA and metagenomic sequencing. However, I am of the opinion that the authors should have processed the samples in parallel (DNase-treated and DNase-non treated) to confirm if indeed the samples were adenovirus negative considering that it is a DNA virus and it might have been removed by nuclease digestion. Our response: The adenovirus real-time PCR had a negative and positive control and these performed well. A statement has been added for the same. The metagenomics protocol may have missed any additional DNA virus but if it was actively replicating there is a good chance of detecting it. Actually our previous metagenomic work despite DNase digestion we detected a number of DNA viruses e.g. bocavirus, herpesvirus 1 and 5, see in <https://www.nature.com/articles/s41598-021-03987-1>. Our assay has still a good chance of detecting DNA virus, probably because of their transcriptomes. None-the less this is still a nice suggestion for taking DNase-treated and DNase-non treated approach for future work although the latter has challenge of a lot of host DNA contamination.

My recommendations regarding this paper are that future studies should include a larger and more diverse sample set from multiple regions to provide a more comprehensive understanding of the outbreak's scope and the virus's epidemiology. Authors need to also incorporate detailed patient demographics, and clinical presentations which will be useful in assessing the impact of the outbreak and inform targeted public health interventions. Our response: We agree. Indeed we now have a study underway looking at a well followed cohort over time is currently evaluating the extent of CA24v locally during the same period and this will provide a much clearer picture on the impact of the outbreak in the community

I concur with the authors that long-term surveillance and studies are necessary to monitor the persistence and evolution of CA24v in the region, as well as to evaluate the effectiveness of public health measures implemented in response to the outbreak. While the findings are significant, providing valuable insights into the aetiology of the AHC outbreak in Kenya, I strongly recommend that the authors consider publishing these results as a brief report instead of a full research article due to the above-mentioned limitations such as small sample size and lack of demographic/clinical data. Our response: Many thanks for the suggestion. We did consider this but the challenge is that such a report ends up even

trimming further the details we have provided which are already considered as limited and more information has been requested. Here we have really attempted providing all details that were within our disposal to enhance readers understanding.

Competing Interests: No competing interests were disclosed.

Reviewer Report 21 February 2025

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Here are few comments from my side:

1. The authors have mentioned that quantitative PCR was used for CVA24v detection but there is no mention of viral load in the results section. Please take a look at the MIQE guidelines for viral load results.
2. Methodology for Rotavirus group A specific quantitative PCR not mentioned.
3. Fluorophore and quencher for probe used in CA24v specific qPCR targetting 5'UTR region is not mentioned.
3. Typographical errors: "conjunctivitis" in place of conjunctivitis
4. The line "All the 13 samples were reanalysed using a CA24v and RVA specific quantitative PCR targeting the NSP3 genome segment" should be reframed as NSP3 in not present in CA24v and thus can create confusion among readers.

the sample size is too small

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Partly

If applicable, is the statistical analysis and its interpretation appropriate?

Partly

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Conjunctivitis, Cataract, Iol, quality of vision

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 21 Mar 2025

Arnold Wasike Lambisia

The authors have mentioned that quantitative PCR was used for CVA24v detection but there is no mention of viral load in the results section. Please take a look at the MIQE guidelines for viral load results. Our response?

2. Methodology for Rotavirus group A specific quantitative PCR not mentioned. Our response: Thanks for highlighting this. This is now added.

3. Fluorophore and quencher for probe used in CA24v specific qPCR targeting 5'UTR region is not mentioned. Our response: Thanks for highlighting. This is now added.

3. Typographical errors: "conjunctivitis" in place of conjunctivitis Our response: This has been corrected on the references

4. The line "All the 13 samples were reanalysed using a CA24v and RVA specific quantitative PCR targeting the NSP3 genome segment" should be reframed as NSP3 is not present in CA24v and thus can create confusion among readers. Our response: The latter part on NSP3 has been deleted to avoid confusion and stated in the methods.

5. the sample size is too small Our response: This is a limitation of this current analysis, which is preliminary. We have since learnt that the virus can be detected in respiratory samples and we are currently working on >100 samples from individuals who attended an outpatient clinic during an outbreak and had symptoms and the virus detected. This bigger analysis will be presented in due course to understand this virus better.

Competing Interests: No competing interests were disclosed.

