Genomic Diversity and Antimicrobial Resistance of *Vibrio cholerae* Isolates from Africa: A

PulseNet Africa Initiative Using Nanopore Sequencing to Enhance Genomic Surveillance

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Abstract

Objectives: *Vibrio cholerae* remains a significant public health threat in Africa, with antimicrobial

resistance (AMR) complicating treatment. This study leverages whole-genome sequencing

(WGS) of *V. cholerae* isolates from Côte d'Ivoire, Ghana, Zambia and South Africa to assess

genomic diversity, AMR profiles, and virulence, demonstrating the utility of WGS for enhanced

surveillance within the PulseNet Africa network.

Methods: We analysed *Vibrio* isolates from clinical and environmental sources (2010–2024)

using Oxford Nanopore sequencing and hybracter assembly. Phylogenetic analysis, multilocus

sequence typing (MLST), virulence and AMR gene detection were performed using Terra,

Pathogenwatch, and Cloud Infrastructure for Microbial Bioinformatics (CLMB) platforms, with

comparisons against 88 global reference genomes for broader genomic context.

Results: Of 79 high-quality assemblies, 67 were confirmed as *V. cholerae*, with serogroup O1

accounting for the majority (43/67, 67%). ST69 accounted for 60% (40/67) of isolates, with eight

sequence types identified overall. Thirty-seven isolates formed novel sub-clades within AFR12

and AFR15 O1 lineages, suggesting local clonal expansions. AMR gene analysis revealed high

resistance to trimethoprim (96%) and quinolones (83%), while resistance to azithromycin,

rifampicin, and tetracycline remained low (≤7%). A significant proportion of the serogroup O1

isolates (41/43, 95%) harboured resistance genes in at least three antibiotic classes.

Conclusions: This study highlights significant genetic diversity and AMR prevalence in African

V. cholerae isolates, with expanding AFR12 and AFR15 clades in the region. The widespread

resistance to trimethoprim and quinolones raises concerns for treatment efficacy, although

azithromycin and tetracycline remain viable options. WGS enables precise identification of

species and genotyping, reinforcing PulseNet Africa's pivotal role in advancing genomic

surveillance and enabling timely public health responses to cholera outbreaks.

Keywords

Cholera, Foodborne infections, PulseNet Africa, Oxford Nanopore Technology Sequencing

Data summary

All supporting data and protocols have been provided within the article or as supplementary data

files. The ONT reads have been deposited under BioProject accession PRJNA1192988, while

the high-quality *Vibrio* spp. assemblies have been shared via figshare (Foster-Nyarko, Ebenezer

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figshare. Dataset. https://doi.org/10.6084/m9.figshare.27941376.v1). Individual accession

numbers for these reads and Biosample IDs are provided in **File S2,** available with the online

version of this article. The accession numbers for the 88 reference genome assemblies included

in our analysis are also provided in **File S3**.

Impact statement

Cholera remains a significant public health challenge in Africa, disproportionately affecting the region due to the ongoing transmission of *Vibrio cholerae* O1 and the emergence of antimicrobial resistance (AMR). This study demonstrates the utility of Oxford Nanopore Technology (ONT) sequencing in providing high-resolution insights into the genomic diversity, transmission dynamics, and AMR profiles of *V. cholerae* isolates across Africa. By generating and analysing whole-genome sequences, we identified novel sublineages, high prevalence rates of AMR genes, and virulence traits critical to cholera pathogenesis. These findings contribute to a deeper understanding of the epidemiology and evolution of *V. cholerae* in Africa, informing targeted intervention strategies.

Furthermore, the study highlights the growing threat posed by AMR among *V. cholerae* isolates, including resistance to key therapeutic antibiotics, such as quinolones and trimethoprim, which could undermine current treatment protocols. Despite this, the absence of resistance to azithromycin and rifampicin among the O1 isolates suggests these drugs may remain viable treatment options, offering a critical avenue for preserving treatment efficacy.

This research also underscores the importance of sustained genomic surveillance, capacity building, and regional collaboration to mitigate the public health impact of cholera and other foodborne pathogens. By leveraging WGS technologies and training initiatives, such as the PulseNet Africa genomics workshop, this study provides a framework for strengthening regional capacities to detect, monitor, and respond to cholera outbreaks and the spread of AMR. These efforts align with the African Union and Africa CDC's strategic priorities on health security and AMR, contributing to improved public health systems and cholera control across the continent.

Introduction

Cholera, caused by toxigenic strains of *Vibrio cholerae* O1 and O139, presents with mild to potentially fatal acute watery diarrhoea (1-3). The current seventh cholera pandemic, primarily due to the O1 biotype El Tor, disproportionately affects Africa, which bears the majority of the global disease burden (2). Despite its longstanding presence since resurging in 1970, the transmission mechanisms of *V. cholerae* in Africa remain poorly understood. This knowledge gap is exacerbated by shifts in antimicrobial resistance (AMR) patterns, moving from traditional antibiotics like streptomycin, chloramphenicol, furazolidone, and trimethoprim-sulfamethoxazole to tetracyclines, fluoroquinolones, and macrolides, climate change, and increased mobility of people, globally (3-7).

Recent genomic analyses have revealed multiple introductions of the *V. cholerae* serogroup O1 biotype El Tor strain from Asia to Africa, with at least 13 distinct sublineages identified since 1970, indicating a continued pattern of introduction from Asia (8-11). Understanding the genomic epidemiology and AMR profiles of *V. cholerae* in Africa is essential for developing effective surveillance and intervention strategies. Conventional methods like culture and antimicrobial susceptibility testing, PCR, and point-of-care testing have been instrumental in providing real-time information on circulating serotypes and AMR patterns in endemic countries in sub-Saharan Africa. However, these methods offer limited resolution and are insufficient for tracking the spread of resistance and understanding the evolutionary dynamics of circulating *V. cholerae* clones. Whole-genome sequencing (WGS) technologies, including Illumina and Oxford Nanopore Technology (ONT), offer improved resolution for characterising outbreak isolates and tracing transmission pathways. By providing comprehensive genomic data, WGS enables a deeper understanding of *V. cholerae* persistence, transmission, and AMR evolution in endemic regions. Such insights are vital for informing more effective cholera control strategies and public health responses.

Given its global distribution, international transmission, and endemicity across most of sub-Saharan Africa, *V. cholerae* has been a priority pathogen for PulseNet Africa since the network's inception in 2010. PulseNet Africa is a regional collaboration of public health laboratories dedicated to tracking food- and water-borne disease outbreaks, including AMR. Alongside *V. cholerae*, PulseNet Africa monitors *Salmonella enterica*, diarrhoeagenic *Escherichia coli*, *Shigella* spp., *Campylobacter* spp., and *Listeria monocytogenes* across 19 African countries (**Figure 1**). It operates within the global PulseNet International network, collaboratively connecting over 108 countries in eight regions to monitor and control foodborne disease outbreaks.

The World Health Organization (WHO) recognises the critical role of WGS in enhancing global public health strategies, particularly for foodborne pathogens and AMR surveillance (12-14). In

the context of foodborne pathogen surveillance, the WHO highlights the utility of WGS in
outbreak investigations, response efforts, and routine monitoring. WGS facilitates precise
pathogen identification, source tracing, and an improved understanding of genetic diversity. This
precision is especially significant within a One Health framework, where human, animal, and
environmental health are interconnected. By enabling more effective tracking of foodborne
diseases, WGS strengthens public health interventions and supports global health security (12,
13).
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Additionally, WGS offers invaluable insights into resistance mechanisms, as well as the
emergence and spread of AMR, informing evidence-based policy development for AMR control
(13). As countries advance toward cholera elimination, WGS analyses can be particularly
impactful, distinguishing between rare, newly detected cases and those arising from previously
circulating endemic strains. Thus, the integration of WGS into national and international
surveillance systems is essential for enhancing precision, enabling swift outbreak responses, and
promoting accurate cross-border data sharing.
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Recently, PulseNet International adopted whole-genome sequencing in place of Pulsed-Field Gel
Electrophoresis (PFGE) and multiple-locus variable number tandem repeat analysis (MLVA) for
molecular sub-typing, owing to the superior resolution of WGS, which has substantially enhanced
genomic epidemiological surveillance and tracking of AMR genes across bacterial populations
(15-18). As molecular capacity continues to expand across the African continent, the
implementation of standardised genomic surveillance for high-priority pathogens has become
increasingly feasible (19). Additionally, the upcoming discontinuation of support for
BioNumerics—the primary software for analysing data generated through PFGE, MLVA, and
WGS—beyond 2024 presents a timely opportunity to evaluate and adopt alternative analysis
tools that can address the diverse needs of the network.
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To strengthen the genomic sequencing capabilities of participating laboratories in PulseNet
Africa, we organised a hands-on genomics workshop focusing on ONT sequencing in
collaboration with the Medical Research Council Unit The Gambia at London School of Hygiene
and Tropical Medicine (MRCG at LSHTM), Theiagen Genomics LLC, the Association for Public
Health Laboratories (USA), and the US Centers for Disease Control and Prevention (CDC). This
workshop, held in July 2024 at the MRCG at LSHTM, provided both wet lab training on ONT
sequencing and dry lab training for genomic data analysis using the Terra platform. Participants
brought DNA from archived, unsequenced isolates identified as *V. cholerae* from outbreaks or
routine surveillance in their countries. These samples were used to train participants on PulseNet
International's protocols for ONT sequencing of food- and water-borne pathogens. The workshop
culminated in collaborative data analysis, where participants interpreted the genomic data to gain

insights into the transmission dynamics and AMR profiles of *V. cholerae* across Africa. We

hypothesised that *V. cholerae* isolates from various African regions would exhibit distinct

transmission events, lineages, and AMR profiles.

Our efforts align with the African Union and Africa CDC's strategic priorities on health security

and AMR. By elucidating the genomic diversity, evolution, and AMR dissemination of *V. cholerae*

in Africa, this study provides crucial insights into the regional and global emergence of multi-

drug-resistant strains. The genomics workshop and this study represent significant steps toward

enhancing the capacity for genomic sequencing of *V. cholerae* and other critical pathogens within

Africa. Additionally, establishing a skilled professional network will help foster ongoing

collaboration and support, potentially contributing to long-term improvements in public health

systems across the continent.

Methods

Archived V. cholerae isolates analysed in this study

We generated WGS data from 104 archived isolates initially identified as *V. cholerae* by

conventional microbiological methods (**File S1**) from four countries: Côte d'Ivoire (n=50), Ghana

(n=19), Zambia (n=29) and South Africa (n=6), between 2010 and 2024 (**Figure 2A**). These

isolates represented unsequenced archived isolates derived from outbreaks or environmental

surveillance (**File S1**). Metadata associated with the isolates included the year of collection and

geographical information (i.e., patient residential province or city) (**File S2**).

Global context isolates

To determine where our isolates sit within the global phylogeny, we included publicly available

sequence data from 88 global reference strains of *V. cholerae* (**File S3**), representing the known

diversity of *V. cholerae* serogroup O1 (8-11, 20-22), collected between 1970 and 2023.

DNA extractions and ONT sequencing

DNA extractions were performed in PulseNet participating laboratories using standard protocols

specific to each laboratory. In Zambia, extractions utilised the Invitek DNA extraction kit (Invitek

Diagnostics, Germany), while isolates from Ghana were extracted using the Qiagen DNeasy

blood and tissue kit (Qiagen Johannesburg). Nucleic acid extractions in Côte d'Ivoire were

performed with the Spin-X viral DNA/RNA extraction kit (SD Biosensor, Republic of Korea), and

in South Africa, the QIAmp DNA mini kit (Qiagen Johannesburg) was used. Library preparation

followed PulseNet International's standard operating procedures using ONT's rapid barcoding kit.

All isolates, except those from South Africa, were sequenced during the training workshop at

MRCG at LSHTM. The libraries were loaded onto 11 MinION R10 flowcells and sequenced on

the GridION, Mk1cs, or Mk1bs platforms. In South Africa, sequencing was conducted at the

National Institute for Communicable Diseases (NICD), and the ONT reads were basecalled using

the same protocol employed at MRCG at LSHTM. The sequences from all locations were

subsequently combined for analysis.

The basecalling utilised ONT's basecaller, Dorado v0.7.2, with the SUPer-accuracy model (SUP)

v4.3.0, via the high-performance computing clusters at MRCG at LSHTM or NICD. Subsequent

bioinformatic analyses were conducted on the Terra platform (https://terra.bio/), the Cloud

Infrastructure for Microbial Bioinformatics (CLIMB) (23) and Pathogenwatch (24).

Bioinformatics analysis

The analysis on Terra utilised the TheiaProk_ONT_PHB v.2.1.0 and TheiaProk_FASTA_PHB

v2.2.0 workflows. Initially, reads were quality-checked with FastQC (25). Assemblies were then

generated on CLIMB using hybracter v0.7.3 (26) via CLIMB (23). The hybracter assemblies were

then uploaded to Terra, where TheiaProk_FASTA_PHB v2.2.0 was deployed to characterise the

genome assemblies. This included speciation with Gambit v1.0.0 (database v2.0.0-

20240628.gdb) (27), identification of *omp*W and t*ox*R loci and *Vibrio* characterisation using

Abricate v1.0.1 (28), multilocus sequence typing using the mlst software v2.23.0

(https://github.com/tseemann/mlst), and plasmid identification with PlasmidFinder v2.1.6 (29).

Next, we uploaded the assemblies to Pathogenwatch (www.pathogen.watch), where a collection

was created and neighbour-joining trees reconstructed using a concatenated alignment of 2,7336

genes which constitute the core gene library for *V. cholerae* in Pathogenwatch. PopPUNK (30)

was deployed to determine the placement of our study isolates as belonging to the current

pandemic lineage using the PopPUNK clusters implemented within Vibriowatch (24). PopPUNK

classifies user-uploaded sequences to one of 41 stable clusters corresponding to the previously

named *V. cholerae* lineages. For example, cluster 1 denotes isolates belonging to the current

pandemic lineage (7PET), clusters 2 and 7 are part of Lineage 3b, while clusters 75 and 76

encompass strains that are part of ELA-1 (24). If a novel lineage is encountered, PopPUNK

assigns cluster VC1870. For ONT assemblies, it is imperative to ascertain that the isolate truly

belongs to a novel lineage and that sequencing errors are not hampering the PopPUNK analysis.

Consequently, for each study isolate typed as VC1780 by PopPUNK or not belonging to

previously characterised lineages, we created a Vibriowatch collection containing the study

isolate and some contextual isolates from the Chun et al. (2009) collection (31), available in

Pathogenwatch (24). This collection encompasses isolates from the 7PET lineage and the other

known *V. cholerae* lineages (31). The neighbour-joining trees, generated by Vibriowatch, were

then inspected to determine the clades the isolate fell into. Additionally, Pathogenwatch calls

AMR genes using an in-house AMR search tool by scanning against a curated AMR gene library

hosted on Pathogenwatch.

To contextualise the AMR genes and mutations found among the *V. cholerae* isolates against

what pertains to the global *V. cholerae* O1 population, we searched for all *V. cholerae* O1

isolates in Pathogenwatch and downloaded the associated AMR genotype predictions and

analysed the resulting data frame using Python's pandas library v2.0.3. via Jupyter Notebook.

We then combined the *V. cholerae* ST69 sequences generated in this study with 88 publicly

available representative sequences from the known 7PET *V. cholerae* sequences and performed

a whole genome alignment of assemblies using the alignment-free mode implemented in the

Split K-mer Analysis (SKA2) v0.3.7 (32)—a rapid and accurate approach which utilises k-mers to

detect variation between samples. Single nucleotide polymorphisms (SNPs) detected by SKA

have been shown to be comparable to those determined by read alignment-based methods such

as Snippy (32-34). A maximum likelihood phylogeny was inferred from the resulting SNP

alignment using RAxML v8.2.12 (35) with the generalised time reversible (GTR) substitution

model and 1000 bootstrap replicates. The phylogenetic tree was visualised using FigTree v1.4.4

and annotated in R v4.1.0 (2021-05-18) with the ggtree package v3.0.4 and Adobe Illustrator

v28.0.

Results

In total, we sequenced 67 clinical and 37 environmental isolates previously identified as *V.*

cholerae. The environmental isolates were collected exclusively from sewage samples in

Abidjan, Côte d'Ivoire, between 2023 and 2024. For the clinical isolates, samples from Côte

d'Ivoire were spread over seven towns or cities; those from Zambia originated from five sub-

counties, while those from Ghana were sourced from the nation's capital, Accra. The isolates

from South Africa were derived from Limpopo and Gauteng (**Figure 2B-C and File S2**).

Since ONT sequencing for *Vibrio* is new to PulseNet International, and QC thresholds for ONT

data are not yet established, we aimed to assess the assembler's performance at varying read

depths. We initially set a minimum read threshold of 5,000 reads, independent of sequencing

depth, hypothesising that fewer reads would be sufficient for high coverage due to the longer

ONT read lengths. All samples met this threshold and were assembled using Hybracter (26). The

resulting assemblies were then examined with Bandage, which showed that samples with read

coverage below 20x generally produced highly fragmented assemblies (>10 contigs) and low

assembly coverage (<20x mean depth), except for three samples (CO42-15, CIV34, and ZA29)

with read coverages of 8x, 13x, and 18x, respectively, which yielded fully circularised

chromosomes. Therefore, in the downstream analysis, we only included samples with fully

assembled chromosomes or a mean read depth and assembly coverage of ≥20x, excluding

those below this threshold. A total of 25 samples, comprising 19 clinical and six environmental

samples, fell below this threshold and were excluded, leaving a final dataset of 79 isolates.

Detailed read and assembly metrics are presented in **Figure S1** and **File S2**.

Of the 25 samples excluded from the final dataset due to insufficient read depth or assembly

quality, 19 were stool samples, and 6 were from sewage. Despite not meeting the QC thresholds,

some samples could still be speciated: 17 were identified as *V. cholerae*, 1 as *Aeromonas*

caviae, and 1 as *Pseudomonas aeruginosa*, while 3 samples remained unidentified, and 3 failed

to assemble altogether. Geographically, these excluded samples were distributed between

Zambia (11), Côte d'Ivoire (11) and South Africa (3).

Of the 79 high-quality assemblies that constitute the final dataset, speciation with Gambit (27)

and Pathogenwatch's Speciator (36) were in agreement and revealed 67/79 (85%) isolates as *V.*

cholerae, 2/79 (3%) each as *V. fluvialis* and *V. navarrensis*, and 1/79 (1%) as *V. furnissii.* The *V.*

furnissii isolate was recovered from a clinical (stool) specimen in Vridi, Côte d'Ivoire, while the *V.*

fluvialis and *V. navarrensis* isolates were all sourced from sewage samples in Abidjan (**File S2**).

All isolates confirmed by WGS as non-cholerae *Vibrio* spp. resulted in complete circularised

assemblies.

The remainder of the isolates typed as non-*Vibrio* species, as follows: *Enterobacter hormaechei*

(4/79, 5%); *Aeromonas enteropelogenes* (2/79, 3%), and *Klebsiella quasipneumoniae* (1/79,

1%).

The 67 isolates confirmed by WGS as *V. cholerae* comprised n=46 from clinical (outbreak) cases

(stool) and n=21 sewage isolates. Of these, 46 had two completely circularised chromosomes

each, comprising n=30 clinical isolates and n=16 from sewage. The assembly metrics are are

depicted in **Figure S1.**

The individual sample accessions for the high-quality *V. cholerae* isolates are provided in **File**

S2, and the *Vibrio* spp. assemblies shared via figshare

(https://doi.org/10.6084/m9.figshare.27941376.v1). The rest of the results focus on the *V.*

cholerae isolates.

Phylogenetic diversity of the study strains

We recovered eight sequence types overall, with ST69 accounting for the majority (40/67, 60%),

followed by non-typeable (12/67, 18%), ST85 and ST75-2LV (2/67, 3% each), ST289, ST832,

ST357, ST366 and ST69-4LV (1/67, 2% each). Six isolates were assigned one or more novel

alleles out of the seven housekeeping genes utilised in the MLST typing, indicating potentially

novel STs.

Most (43/46, 93%) of the clinical isolate genomes were identified as *V. cholerae* serogroup O1
and biotype El Tor, consistent with the serological results. The majority of *V. cholerae* O1 isolates
belonged to ST69 or its close variant ST69-4LV and were assigned to the VC1 cluster by
PopPUNK, indicating their association with the current pandemic lineage. Notably, two
exceptions were observed: the ST75-2LV isolates from South Africa, which clustered within the
pre-7PET clade (**Figure S2**). This clade is thought to represent close relatives of the most recent
common ancestor of the 7PET clade (24). The nearest relative to the two ST75-2LV strains was
isolate 2740-80, a US Gulf Coast *V. cholerae* O1 clone previously associated with the 2018–
2020 cholera outbreak in South Africa (37). In addition, two non-O1 clinical isolates ('CIV50' and
'CIV55'), collected from Abobo-Doumé and Williamsville, Côte d'Ivoire, respectively, were
identified as belonging to ST85 and were assigned to the VC995 cluster. A single environmental
isolate from ST366 was assigned to VC506. The remaining non-O1 environmental isolates were
grouped into VC1870 by PopPUNK, further underscoring their genomic diversity.

The phylogenetic analysis with representative global reference strains revealed distinct sub-
lineages arising from local clonal expansions of AFR9, AFR12 and AFR15 among the *V.
cholerae* O1 ST69 strains. Specifically, isolates '17-14' and 'C413-14', collected in Accra in 2014,
along with isolate CIV57 derived from Adiaké, Côte d'Ivoire, in 2012, clustered with reference
genomes belonging to T9/AFR9, indicating genetic similarity to these known *V. cholerae* O1
strains. Likewise, n=22 isolates from Ghana (2010 – 2015) and Côte d'Ivoire (2012 – 2014)
formed novel sub-clades within the AFR12 lineage (**Figure 3**), indicative of local clonal
expansions of this lineage in these two countries. This sub-lineage was initially identified in Togo
and thought to have been directly imported from South Asia (11). Similarly, the O1 isolates
collected from central Lusaka, Nangoma and Kanyama in Zambia between 2023 and 2024
formed novel sub-clusters within the recently defined sub-lineage AFR15, which caused a large
outbreak in South Africa between 2022 and 2023 (8), suggesting potential uncharacterised clonal
expansions of this lineage within the *V. cholerae* O1 serogroup and underscoring the genetic
diversity among our study isolates.

Prevalence of acquired resistance genes and resistance mutations
The prevalence of genes encoding resistance to various antibiotics among the *Vibrio cholerae*
study isolates is depicted in **Figure 4** and **Figure 5**. Trimethoprim resistance genes exhibited the
highest prevalence, with 64 out of the 67 isolates (96%) harbouring at least one of four variants
of the *dfr*A gene (**Figure 4** and **Figure 5**). *dfr*A1 occurred predominantly in the clinical isolates
(40/46, 93%) (**Figure 4**). The environmental *V. cholerae* isolates, on the other hand, mainly
harboured *dfr*A31 (7/24, 29%), along with *dfr*A15 (4/24, 17%) and *dfr*A1 (2/24, 8%) (**Figure 5**).
Conversely, some clinically relevant antibiotics demonstrated little or no resistance determinants

among the study isolates. Notably, no determinants associated with azithromycin or rifampicin

resistance were observed. Low prevalence rates were also seen for the antiseptic resistance

gene *qac*Edelta, which occurred exclusively among the environmental strains (4/24, 16% of

isolates). Similarly, tetracycline resistance genes occurred only among the environmental

isolates, echoing findings from Algeria, Central African Republic, Kenya and Malawi, indicating

widespread susceptibility of *V. cholerae* O1 isolates to tetracyclines (38-41). The genotypes

included *tet*(C) in 4% of the environmental isolates (1/24), *tet*(G) in 13% (3/24), and *tet*(59) in 4%

(1/24). The ampicillin resistance gene, bla_{CARB-2} gene, was found in the sewage isolates only

(4/24, 12%).

The preponderance of the resistance determinants and mutations occurred among the *V.*

cholerae O1 isolates (**Figure 4**), consistent with findings from other studies (38-42). For example,

resistance determinants for chloramphenicol (*cat*B9 and *flo*R), aminoglycosides (*str*A and *str*B)

and sulfamethoxazole (*sul*2) occurred in ≥88% (38/43) of the O1 strains but were detected in 4-

16% of the non-O1 isolates (*cat*B9, 1/24, 4%; *flo*R, 3/24, 12%; *str*A, 3/24, 12%; *str*B, 2/24, 8%;

*sul*1, 4/24, 16%; respectively). Conversely, the encoding resistance to ampicillin was identified in

environmental isolates only (3/24, 12%), as was the bla_{CMY-4} gene encoding resistance to third-

generation cephalosporins, which occurred in a single environmental isolate (1/24, 4%) (**Figure**

5).

Besides these, the *var*G resistance gene, a putative Ambler class B metallo-beta-lactamase

found on the antibiotic resistance *var* regulon in *V. cholerae*, along with an antibiotic efflux pump

(43), was present in 98% of the clinical *V. choloerae* O1 isolates (42/43) and 29% (7/24) of the

environmental (non-O1) strains. However, we could not find much evidence for phenotypic

carbapenem resistance due to *var*G in the literature; thus, further investigation is warranted to

confirm this gene as a functional carbapenemase (43-45).

In addition to resistance genes, specific mutations conferring resistance to other antibiotic

classes were identified. The *gyr*A_S83I and *par*C_S85L mutations, associated with quinolone

resistance, were detected in 88% (38/43) of the serogroup O1 isolates. These isolates belonged

to the current pandemic (7PET) clade and were distributed across the AFR12 and AFR15

lineages, consistent with the fixation of this trait in strains from these lineages (42). By contrast,

the remaining serogroup O1 isolates, which lacked these fluoroquinolone resistance mutations,

comprised three ST69 strains from the AFR9 lineage and two ST75-2LV clones from the pre-

7PET clade.

Among non-O1 isolates, the *gyr*A_S83I and *par*C_S85L mutations were present in 50% (12/24)

and 33% (8/24) of isolates, respectively. Resistance to furazolidone was mediated by the

nfsA_R169C and nfsB_Q5* mutations, both of which were detected exclusively in the O1 isolates (41/43, 95%, all belonging to ST69) (**Figure 4** and **Figure 5**), indicating the fixation of these mutations in the O1 ST69 population.

Nearly all the *V. cholerae* O1 isolates (41/43, 95%) harboured resistance genes in at least three antibiotic classes, excluding *var*G (carbapenems). The high levels of resistance to critical antibiotics, particularly ciprofloxacin and nalidixic acid, raise significant concerns for treatment efficacy. However, the absence of resistance to tetracycline, azithromycin, and rifampicin among the V. cholerae O1 isolates suggests these antibiotics may remain effective treatment options.

Our findings mirrored a high prevalence of quinolone and nitrofuran resistance mutations and several resistance genes in the global population of *V. cholerae* O1 isolates (**File S4**). An analysis of n=4,858 *V. cholerae* O1 genomes hosted in Pathogenwatch (24) indicates that mutations *nfsB_Q5* and *nfsA_R169C*, conferring resistance to nitrofurans, are present in 70% of O1 isolates globally (3419/4858 and 3408/4858, respectively). Similarly, the *gyrA_S83I* and *parC_S85L* mutations occur in 63.96% (3107/4858) and 56.69% (2754/4858) of global isolates, respectively, reflecting the prevalence observed in our study where *gyrA* and *parC* mutations were present in 88% (38/43) of O1 isolates.

Our findings are consistent with the global prevalence of resistance genes, with *var*G detected in 99.77% (4847/4858) of all O1 strains, supporting the hypothesis that this gene is intrinsic to the *V. cholerae* population. Other notable resistance genes, such as *dfr*A1, *cat*B9, and *sul*2, encoding resistance to trimethoprim, chloramphenicol, and sulfamethoxazole, respectively, also exhibited similar global frequencies (*dfr*A1: 65.87% or 3200/4858; *cat*B9: 99.61% or 4839/4858; *sul*2: 63.59% or 3089/4858).

These findings highlight the widespread distribution of resistance traits in the global *V. cholerae* O1 population, likely driven by selective pressures and the dissemination of resistant lineages, particularly in clinical settings.

Prevalence of virulence traits
Several virulence genes appeared to be conserved across the study isolates (**Figure 6**). These included the adhesion-associated genes, *rpo*S, *omp*U and the MSHA pilus; the haemagglutinin gene *hap*A, known for its cytotoxic and mucinolytic activities (46); the pore-forming toxin, *hly*A (haemolysin) (47); and *tox*R, the transmembrane transcription factor that regulates the production of virulence factors in *V. cholerae* (48), indicative that they were core to the species.

Besides these, several virulence traits appeared to be fixed among the clinical *V. cholerae* O1

study isolates. For example, the cholera toxin genes *ctx*A and *ctx*B, which produce the 'rice-

water' stools characteristic of cholera (49, 50); the toxin gene *ace*; the colonisation operon *acf*A,

*acf*B, *acf*C, *acf*D; the toxin co-regulated pilus (TCP) cluster, associated with colonisation in

humans and animals (51), and the toxin gene *zot* occurred in >93 % (40/43) of the *V. cholerae*

O1 isolates but were not detected among any of the non-O1 isolates. By contrast, the cholix toxin

gene, *chx*A (an exotoxin) (52), was found in 8% (2/24) of the environmental isolates but was not

found among any of the clinical isolates. A previous study found *chx*A in both O1 (clinical) and

non-O1 (environmental) strains (53). The complete list of virulence traits detected among the

study *Vibrio* isolates is provided in **File S5**.

Discussion

This study demonstrates the utility of Oxford Nanopore Technology (ONT) sequencing for

enhancing genomic surveillance of *V. cholerae* in Africa, a critical capability for networks like

PulseNet Africa. By applying ONT sequencing, we were able to generate high-resolution data on

the genetic diversity, AMR profiles, and virulence traits of *V. cholerae* isolates from multiple

African countries. This approach has significant potential for strengthening regional surveillance

efforts for foodborne pathogens, including *V. cholerae*, as well as other critical pathogens under

PulseNet Africa's remit. The ability to rapidly generate and analyse whole-genome sequences

using ONT in resource-limited settings could enhance the early detection of outbreaks, track the

spread of AMR, and improve public health responses across the continent.

Among the clinical isolates analysed, three non-O1 strains were retrieved from cases presenting

with cholera-like symptoms. In addition, several study isolates were misidentified as *V. cholerae*,

including non-*Vibrio* species such as *K. quasipneumoniae*, *E. hormaechei*, and *A.*

enteropelogenes. These findings underscore a significant diagnostic challenge: pathogens

causing cholera-like disease are frequently misidentified as *V. cholerae* by conventional

microbiological methods. Previous studies in Africa and elsewhere have suggested that certain

enteric bacteria can cause cholera-like diarrheal infections, leading to their misattribution to *V.*

cholerae during cholera outbreaks (54-57). This issue is further underscored by findings from a

large cholera outbreak in Malawi, where Chaguza et al. (2024) identified non-*V. cholerae* species

in approximately 28% of cases (19 out of 68) (39).

The identification of clinically relevant *Vibrio* species, such as *V. fluvialis*, *V. furnissii*, and *V.*

navarrensis—all of which cause gastrointestinal illness (58)—is particularly noteworthy in the

context of horizontal gene transfer among *Vibrio* species (59). For instance, *V. mimicus*, though a

non-*cholerae* species, can harbour the cholera toxin (*ctx*) gene (60), highlighting the potential for

genetic exchange that may influence virulence. Similarly, *V. fluvialis* has been shown to possess

an El Tor-like hemolysin (*hly*A), a gene associated with pathogenicity (61), underscoring the

importance of monitoring these species for genetic traits relevant to outbreaks and public health.

From this study, the *V. furnissii* isolate was recovered from a clinical stool specimen, while *V.*

fluvialis and *V. navarrensis* isolates were all sourced from sewage samples. These findings

emphasise the necessity of accurate genomic characterisation and expanded surveillance for

Vibrio species, especially given the challenges of speciation due to phenotypic heterogeneity

even within a single *Vibrio* species. Genomic surveillance will be critical to improving pathogen

detection, understanding their evolutionary dynamics, and mitigating risks posed by emerging

strains.

An earlier survey of global *V. cholerae* diversity by Weill et al. (2017) (11) included strains from

Côte d'Ivoire (n=29, spanning 1970–2006), Zambia (n=26, spanning 1996–2012), and Ghana

(n=8, spanning 1970–2014). Our study extends this timeline, including isolates collected between

2010 and 2024, and provides new insights into the genomic diversity of *V. cholerae* O1 in these

regions.

The phylogenetic analysis uncovered significant genetic diversity among the isolates, with

several forming novel clades, particularly within the *V. cholerae* O1 serogroup. Most clinical

isolates clustered within the pandemic 7PET lineage, consistent with previous studies on *V.*

cholerae transmission in Africa (4, 8, 9, 11, 15, 41). However, the discovery of distinct novel

sublineages among clinical O1 isolates (35/45, 77.8%) underscores the potential for ongoing,

uncharacterised transmission events or the importation of new strains into the region. Consistent

with recent reports from South Africa, the two ST75-2LV strains from our study clustered closely

with isolates from the pre-7PET clade. The closest relative to these strains was the US Gulf

Coast *V. cholerae* O1 clone (37), highlighting the continued dominance of ST75 in cholera cases

reported in South Africa. The environmental strains also displayed considerable diversity, with

the 24 isolates being spread over five distinct STs and six potentially novel STs. Such diversity

underscores the importance of continuously monitoring these populations to detect emergent

strains that may acquire virulence factors or resistance genes.

The clustering of non-O1 isolates into separate groups, particularly those recovered from sewage

samples, indicates a divergence in environmental strains from those driving outbreaks. At least in

our study, environmental strains do not appear to seed outbreaks, as they are genetically quite

divergent from the clinical strains, although they harbour some key virulence traits. Further

investigation into the role of environmental reservoirs as potential contributors to future outbreaks

and their broader role in cholera epidemiology is warranted (62-65).

The high prevalence of AMR genes in the study isolates is concerning, especially given the

widespread presence of resistance determinants for clinically essential antibiotics such as

trimethoprim, ciprofloxacin, and nalidixic acid. The near-universal presence of *dfrA* alleles and

high prevalence of quinolone resistance mutations (*gyr*A_S83I and *par*C_S85L) suggest that

these drugs may be increasingly ineffective for cholera treatment in the region. This finding

mirrors global trends of rising resistance to quinolones and other frontline antibiotics (4, 11, 66),

which may complicate treatment strategies and necessitate a shift in therapeutic approaches.

The detection of AMR genes in all O1 isolates, with each exhibiting resistance to at least three

classes of antibiotics, is consistent with findings from other African studies (38-42) and the global

V. cholerae O1 populations and raises concerns about the continued efficacy of current

treatment protocols. While no resistance determinants were identified for azithromycin and

rifampicin, the growing resistance to other antibiotics used in cholera treatment, such as

tetracyclines and quinolones, suggests that alternative strategies must be developed. The

absence of azithromycin resistance among these isolates offers a potential avenue for

maintaining treatment efficacy, as this drug may remain a viable option for first-line treatment (4,

6).

The stark contrast in resistance profiles between O1 and non-O1 isolates further emphasises the

complexity of cholera epidemiology in Africa. O1 isolates exhibited significantly higher levels of

AMR, with many harbouring resistance determinants to multiple classes of antibiotics. This

difference may reflect the clinical settings from which O1 isolates were derived, as these strains

are more likely to have been exposed to selective pressures from antibiotic use in humans. In

contrast, non-O1 isolates, particularly those from environmental sources, displayed

comparatively lower levels of resistance, which could suggest reduced exposure to antibiotics in

these reservoirs. However, the potential for environmental strains to acquire resistance genes

through horizontal gene transfer cannot be overlooked and requires ongoing monitoring. While

the environmental strains displayed fewer resistance genes compared to clinical isolates, there

were overlaps in certain determinants; e.g., the quinolone mutations, *gyr*A_S83I and *par*C_S85L,

occurred in 50% and 33% of the environmental strains, respectively. This suggests some shared

AMR determinants between the two groups, which is crucial to further investigate the direction of

AMR transmission within the environment and the potential role of environmental strains in the

spread of resistance.

Although more virulence genes were found in the clinical strains, with several being fixed in the

population but absent from the environmental strains, there were key overlaps in virulence genes

among both O1 and non-O1 strains, warranting continuous monitoring. The high prevalence of

virulence genes among the O1 isolates confirms their pathogenic potential and role in driving
cholera outbreaks in the region.
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Limitations
A major limitation of this study is that the sample collection relied on available archived and
viable isolates from four countries. As a result, the collection may not fully represent the diversity
of circulating *Vibrio cholerae* strains in the region during the study period. The lack of phenotypic
antimicrobial susceptibility test data is another limitation. Although we were able to analyse the
genotypic profiles of AMR, phenotypic data would have provided direct evidence of resistance
patterns in the isolates. Unfortunately, logistic constraints did not allow us to conduct phenotypic
testing, which limits the study's ability to fully assess the clinical implications of the detected
resistance genes.
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Conclusion
Our study demonstrates the value of using WGS to uncover the genomic diversity, AMR profiles,
and virulence traits of *V. cholerae* strains in Africa. Further work is needed to develop
standardised QC metrics and standard operating procedures for handling ONT sequencing data
within PulseNet Africa and its sister networks. Establishing these protocols will ensure data
consistency, reliability, and comparability across labs, thereby strengthening the regional
genomic surveillance framework for *Vibrio cholerae* and other pathogens.
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592 The identification of clonal expansion within certain lineages, characterised by high levels of
593 AMR genotypes in clinical isolates, provides essential information for improving cholera
594 surveillance and response strategies. Given the high prevalence of resistance to critical
595 antibiotics such as trimethoprim and quinolones and the potential for further AMR evolution, it is
596 imperative to reassess treatment guidelines and enhance genomic monitoring.
597

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Data Collection – Conducting experiments, collecting samples, gathering or analysing data: SAB,

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Methodology – Development or design of the methodology; selecting and designing the research

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Conflicts of interest

The authors declare they have no conflicts of interest.

Ethical statement

The inclusion of country-specific data in this study was approved by the institutional review

boards and respective ministries of health in the four countries that contributed samples.

Informed patient consent was waived as the samples were collected as part of routine diagnostic

procedures. All patient data associated with these isolates were anonymised to ensure no

possibility of patient identification based on age, sex, or hospital-related information. This

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Figures and Legends

Figure 1. PulseNet International Regions and PulseNet Africa Member Countries. (**A**) A depiction of PulseNet International Regions, colour coded as follows: blue, European Food and Waterborne Disease Network; orange: PulseNet Africa; green: PulseNet Asia Pacific; purple: PulseNet Canada; brown: PulseNet Latin America and Caribbean; grey: PN Middle East; light yellow: PulseNet International Partners; light cyan: PulseNet Eastern Europe and Central Asia. The participating countries representing Africa, Asia Pacific, Eastern Europe and Central Asia, the European Food and Waterborne Disease Network, Latin America and Caribbean, Middle East and PulseNet International Partners are denoted in the corresponding colour-coded bubbles. (**B**) This panel zooms in on the PN Africa region and highlights its member countries.

Figure 2. Geographical distribution, collection year, and sample source of the isolates analysed in this study.
(**A**) Geographical distribution of the isolates analysed in this study. The map highlights the locations where the samples were collected. (**B**) The number of isolates collected per year is displayed, showing the temporal distribution of sample collection across different years per sampling site, as depicted in the legend. (**C**) The sample sources are categorised and plotted to represent the proportion of samples collected from outbreak (Stool) or non-outbreak (Sewage) situations.

AFR13 AFR11 AFR12 AFR11

AFR14

AFR15 clonal expansion

● Zambia (n=15)
    2023-2024

AFR12 clonal expansion

● Ghana (n=16), 2010 - 2015

● Côte d'Ivoire (n=6), 2012 - 2014

AFR2

AFR1

AFR10

AFR3

AFR9

● Ghana (n=2), 2014

● Côte d'Ivoire (n=1), 2012

AFR4

AFR7

AFR8

AFR6

AFR5

Figure 3. A Maximum-Likelihood tree depicting the population structure of the *V. cholerae* ST69 O1 study isolates (n=40), along with 88 publicly available genomes representing the known diversity of *V. cholerae* O1. The phylogenetic tree was reconstructed using RAxML v8.2.12 (Reference #35) with 1000 bootstrap replicates, based on a reference-free whole genome alignment generated with SKA2 (Reference #32). The branch lengths indicate genetic distances between samples. The clades are highlighted and annotated to show the previously known lineages (e.g., AFR1, AFR2), with the study samples distinguished from the representative reference genomes by the black circular tips (the reference genomes are tip-less, each colour denoting a unique *V. cholerae* O1 lineage. Where they occur, the distribution of study isolates found in each lineage is displayed   next to the lineage name. Distinct clonal expansions with AFR12 and AFR15 arising from this study are denoted in red.

Figure 4. A neighbour-joining phylogenetic tree of the study *V. cholerae* ST69 serogroup O1 isolates (n=40) with antimicrobial resistance genes and virulence annotations. The phylogenetic tree depicts the evolutionary relationships among the study isolates, as determined by phylogenetic inference. The tree was reconstructed via Pathogenwatch (Reference #24), based on a concatenated alignment of 2,736 genes (3,075,173 bp) representing the core gene library for *V. cholerae* in Pathogenwatch. Each tip of the tree denotes a unique isolate, coloured by the geographic origin or source of the isolate (displayed in the legend). The heatmap visualises the presence of acquired antimicrobial resistance genes (raspberry), resistance mutations (blue raspberry) and virulence factors (light pink). The resistance genes, grouped by their classes, are as follows: carbapenems (*var*G), chloramphenicol (*cat*B9, *flo*R), aminoglycosides (*str*A, *str*B) and trimethoprim (*dfr*A1 and *dfr*A_new). Resistance mutations were as follows: quinolone resistance mutations (*gyr*A_S83I and *par*C_S85L) and nitrofuran (*nfs*A_R169C and *nfs*B_Q5*).

Figure 5. A neighbour-joining phylogenetic tree of the study *V. cholerae* non-ST69 isolates (n=27) with antimicrobial resistance genes and virulence annotations. The phylogenetic tree depicts the evolutionary relationships among the study isolates, as determined by phylogenetic inference. The tree was reconstructed via Pathogenwatch (Reference #24), based on a concatenated alignment of 2,736 genes (3,075,173 bp) representing the core gene library for *V. cholerae* in Pathogenwatch. Each tip of the tree denotes a unique isolate, coloured by the geographic origin or source of the isolate (displayed in the legend). The heatmap visualises the presence of acquired antimicrobial resistance genes (raspberry) and resistance mutations (blue raspberry). The resistance genes, grouped by their classes, are as follows: penicillins (*bla*CARB-2), carbapenems (*var*G), third-generation cephalosporins (*bla*CMY-4), chloramphenicol (*cat*B9, *flo*R), sulphonamides (*sul*1, *Sul*2), aminoglycosides (*aad*A1, *aad*A2, *str*A, *str*B), tetracyclines (*tet*(C), *tet*(G), *tet*(59)), trimethoprim (*dfr*A1, *dfr*A15, *dfr*A31), and antiseptic (*qa*CEdelta). Resistance mutations were detected for quinolones as follows: (*gyr*A_S83I and *par*C_S85L

Figure 6. Binary Heatmap showing the presence and absence of virulence traits among the study *V. cholerae* isolates. The heatmap displays the presence (raspberry) and absence (soft cyan) of specific virulence genes across the study isolates. Each row represents a sample, and each column represents a gene of interest, as identified via Pathogenwatch and TheiaProk.

The resistance genes, by their classes, are as follows: *rpo*S, a stress gene involved in adhesion; *omp*U, an outer membrane protein also involved in adhesion; MSHA Pilus, a pili structure that promotes adhesion to host surfaces; *hap*A, a haemagglutinin/protease gene that facilitates bacterial invasion and immune evasion; *hly*A, a haemolysin gene that contributes to cytotoxicity; *tox*R, a transmembrane transcriptional activator that regulates the production of virulence factors, including cholera toxin; *ctx*A and *ctx*B, the cholera toxin genes responsible for cholera toxin production; ace, a toxin gene associated with cholera enterotoxin; *acf*A, *acf*B, *acf*C, and *acf*D, colonisation factor genes necessary for intestinal colonisation; *zot*, a toxin gene that increases intestinal permeability; *chx*A, a toxin gene; and *vas*X, a gene encoding a toxin involved in the type VI secretion system, aiding bacterial virulence.

Figure S1: Distribution of Read and Genome Assembly Metrics

Panels A–D: Violin plots showing the distribution of genome assembly metrics across *Vibrio cholerae* samples (n=67), as follows: **A.** Genome length distribution (in base pairs). **B.** Number of contigs distribution. **C.** N50 distribution. **D.** GC% distribution across genome assemblies.
Panels E–H: Read quality and coverage metrics for ONT sequencing: **E.** Histogram showing the distribution of read N50 values. **F.** Read N50 boxplot summarising the range and outliers in read N50 values. **G.** Histogram depicting the average read depth distribution. **H.** Boxplot illustrating the spread and outliers in average read depth values.

Figure S2. A Neighbour-Joining tree illustrating the phylogenetic placement of the study isolates ST-69-4LV and ST-75-2LV. The tree was reconstructed using Vibriowatch (Reference #24) and incorporates the collection of isolates published by Chun et al. (Reference #29). Study isolates are represented by circular tips with red labels, while reference isolates from the Chun collection are represented by square tips and black labels.

The soft brick red dashed rectangle highlights isolates belonging to the current global "7th pandemic" (7PET) clade, whereas the teal rectangle marks the broader Phylocore Group 1 (PG-1) clade. Notably, the two ST-75-2LV study isolates cluster closely with NCTC_8457, MAK757, and BX330286, which are sometimes referred to as 'pre-7PET' isolates.

Figure S3: Prevalence of resistance genes and mutations in O1 vs non-O1 isolates. (**A**) The bar plot shows the prevalence of resistance genes across O1 and non-O1 isolates. The blue bars represent the prevalence of resistance genes in O1 isolates, while the orange bars represent the prevalence in non-O1 isolates. The genes are grouped by antibiotic resistance classes, including penicillins (*blaCARB-2*), carbapenems (*var*G), third-generation cephalosporins (*bla*$_{CMY-4}$), chloramphenicol (*cat*B9, *flo*R), aminoglycosides (*aad*A1, *aad*A2, *str*A, *str*B), tetracyclines (*tet*(C), *tet*(G), *tet*(59), and trimethoprim (*dfr*A1, *dfr*A15, dfrA31, *dfr*A_new).

(**B**) The bar plot shows the prevalence of key mutations associated with antibiotic resistance in O1 and non-O1 isolates. The blue bars represent the prevalence in O1 isolates, and the orange

bars represent non-O1 isolates. The mutations include those associated with quinolone resistance (*gyr*A_S83I, *par*C_S85L) and furazolidone resistance (*nfs*A_R169C, *nfs*B_Q5*). Data labels indicate the precise prevalence percentages.