nature portfolio

Peer Review File

An unusual two-strain cholera outbreak in Lebanon, 2022-2023: a genomic epidemiology study

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
It was a pleasure to review the manuscript by Fayad, Rafei, Njamkepo, Ezzedine and colleagues on the cholera outbreak in Lebanon in 2022–2023. This work describes the genomic characteristics of the Vibrio cholerae strains associated with the outbreak. The key finding of this manuscript is that the outbreak was caused by multiple serogroup O1 Ogawa strains belonging to wave 3 of the 7th pandemic El Tor (7PET) lineage with different antimicrobial resistance profiles. These strains clustered with strains associated with outbreaks elsewhere, mainly in Asian countries. This work provides much-needed genomic information to understand further the strains that caused the resurgence of cholera in Lebanon, a country whose last notable cholera outbreak before the 2022–2023 outbreak occurred in 1993.

The manuscript is clear and well-written, and the bioinformatics methods used for the analysis appear sensible and justified. While the manuscript's conclusions are well-supported by the current dataset, sequencing a larger collection of isolates could have allowed for a more detailed within-country analysis to understand the geographical distribution of the two different 7PET strains circulating in Lebanon. Overall, this is an excellent and timely study, considering recent reports of cholera outbreaks in several countries. I have a few specific minor comments below.

Minor comments:

1. Consider adding a concluding sentence at the end of the abstract.
2. I understand why the global 7PET phylogeny was rooted on the A6 genome. It would be great to briefly mention this genome is suitable for rooting the 7PET phylogeny.
3. The authors mention that the finding of multiple 7PET strains circulating in the same location is unusual, as pointed out in the manuscript. A recent study in Malawi found both ST69 and non-ST69 during the 2022–2023 outbreak in Malawi (https://www.medrxiv.org/content/10.1101/2023.06.08.23291055v1). Could you also comment in the introduction or discussion whether outbreaks caused by a single 7PET and non-7PET strains are also uncommon?
4. Consider discussing the introduction times of the two 7PET strains identified in Lebanon. For example, is it likely that the strains were introduced around the same time? If one of the strains was introduced much earlier than the other, the authors should speculate why it did not cause any outbreak before the 2022–2023 outbreak.
5. Some discussion focusing on the differences in the prevalence of the two strains would be great. Did the predominant strain harbour unique genomic features that would make it more virulent and transmissible than the minority strain? Or was the observed number of cases primarily due to the differences in the affected populations in the South of Lebanon and elsewhere?

Reviewer #2:
Remarks to the Author:
The authors have done a comprehensive genome-wide analysis of 34 isolates randomly sampled from 671 V. cholera isolates that caused outbreak after 30 years in Lebanase during October'2022 to January '2023. Since Lebanon is not endemic to cholera and the exclusivity of the V. cholera strains in this outbreak from their previous two episodes indicates travel associated transmission.
The very containment of the infection with vaccination highlights the interlinked immunity mediated protection over the transmission of V. cholera in this outbreak.
The study indicates the presence of ctxB7 allele and the characteristic tcpACIRS101and VSP-IIΔ in all the 34 WGS variants. These variants emerged with the seventh pandemic V. cholerae El Tor (7PET) at the third wave and is associated with high Cholera toxin synthesis and high intercontinental

transmission potency.
We also appreciated the authors WGS and phylogeny based approach to unlock the independent introduction of the two variants, the low AMR and MDR strains attributed to this outbreak.
The manuscript is well drafted and appreciated, however we have the following concerns to be addressed before it is further processed.
The authors may justify for sampling a very size small size (34/671) from the laboratory confirmed cases for WGS analyses. We also wonder whether the genomic analyses be extrapolated to categorize the remaining phenotypically categorized V. cholerae strains as all the 671 were well grouped into two categorized low AMR and extensive MDR strains.
The frequent usage of the terminology of narrow resistance and extended MDR in the manuscript is confusing. We thus suggest the authors to categorize the strains as either antimicrobial resistant (AMR) /multidrug resistant (MDR) and extensive drug resistant XDR as addressed universally throughout this manuscript for better clarity.
Subsequently, the authors may define the criteria for MDR/ extended MDR in the methodology.
We suggest for time reconstruction from the patient meta data to reconfirm the independent introduction of two different strains in Lebanon as against the other hypothesize of convergent evolution of the MDR strain from the predominant narrow resistant strains.
Line 82: Does the second case connection with the first case?

The authors may provide a geographical of Lebanon, to better understand the contents provided in "72-92"; especially the locations of South of Lebanon (Tyr) and Beqaa, from where the MDR strains were predominantly isolated. We also insist relevent correction in Fig.1 b.

Fig. 1b: The authors mention that the last reported case were in January'2023(Line No. 94). The cumulative suspected cases in the figure indicates logarithmic increase against the subsequently decreasing confirmed cases. We suggest an explanation for this.

Details regarding how many out of the 671 isolates were sent to AUB and how many were stored at LMSE. (Line 146-147) is confusing.
The authors may provide information on how was the O antigen determined phenotypically for the 671 so that the categorization of the O1 biotype was confirmed.
Line 324: Focusing on sub-genomic regions like mobile elements to understand cholera's spread has made El Tor isolates from the 7th pandemic appear more diverse than they truly are, compromising accurate molecular epidemiological mapping. In this context, we appreciate the author's approach of using SNV in place of SNP (which requires at least 1% prevalence of the variant(s). However, we insist the authors provide details of the bioinformatics methodologies to evaluate SNV and as well as construction of the phylogenetic tree.
Fig.3: We insist on including AFR15 in the color code. May clarify details on how both the outbreak groups were placed against the AFR13 and in parallel to the Middle East region. Line 365-367 conveys that Lebanon isolates belonged to AFR15 sublineage.

Authors: We would like to thank the reviewers for their many insightful comments. We address these comments, point by point, below.

Reviewer #1

It was a pleasure to review the manuscript by Fayad, Rafei, Njamkepo, Ezzedine and colleagues on the cholera outbreak in Lebanon in 2022–2023. This work describes the genomic characteristics of the Vibrio cholerae strains associated with the outbreak. The key finding of this manuscript is that the outbreak was caused by multiple serogroup O1 Ogawa strains belonging to wave 3 of the 7th pandemic El Tor (7PET) lineage with different antimicrobial resistance profiles. These strains clustered with strains associated with outbreaks elsewhere, mainly in Asian countries. This work provides much-needed genomic information to understand further the strains that caused the resurgence of cholera in Lebanon, a country whose last notable cholera outbreak before the 2022–2023 outbreak occurred in 1993.

The manuscript is clear and well-written, and the bioinformatics methods used for the analysis appear sensible and justified. While the manuscript's conclusions are well-supported by the current dataset, sequencing a larger collection of isolates could have allowed for a more detailed within-country analysis to understand the geographical distribution of the two different 7PET strains circulating in Lebanon. Overall, this is an excellent and timely study, considering recent reports of cholera outbreaks in several countries. I have a few specific minor comments below.

Minor comments:

1. Consider adding a concluding sentence at the end of the abstract.

Authors: We have modified the abstract to allow the addition of a concluding sentence while respecting the word-count limit (200 words). It now reads: *"Cholera is a life-threatening gastrointestinal infection caused by a toxigenic bacterium, Vibrio cholerae. After a lull of almost 30 years, a first case of cholera was detected in Lebanon in October 2022. The outbreak lasted three months, with 8,007 suspected cases (671 laboratory-confirmed) and 23 deaths. We used phenotypic methods and microbial genomics to study 34 clinical and environmental Vibrio cholerae isolates collected throughout this outbreak. All isolates were V. cholerae O1, serotype Ogawa strains from wave 3 of the seventh pandemic El Tor (7PET) lineage. Phylogenomic analysis unexpectedly revealed the presence of two different strains of the seventh pandemic El Tor (7PET) lineage. The dominant strain had a narrow antibiotic resistance profile and was phylogenetically related to South Asian V. cholerae isolates and derived African isolates from the AFR15 sublineage. The second strain was geographically restricted and extensively drug-resistant. It belonged to the AFR13 sublineage and clustered with V. cholerae isolates collected in Yemen. In conclusion, the 2022-2023 Lebanese cholera outbreak was caused by the simultaneous introduction of two different 7PET strains. Genomic surveillance with cross-border collaboration is therefore crucial for the identification of new introductions and routes of circulation of cholera, improving our understanding of cholera epidemiology."*

2. I understand why the global 7PET phylogeny was rooted on the A6 genome. It would be great to briefly mention this genome is suitable for rooting the 7PET phylogeny.

Authors: We have added the following explanation to the Methods section: *"This global tree was rooted on the A6 genome — the earliest and most ancestral seventh pandemic isolate, collected in Indonesia in 1957 (ref.[11]) — and visualised with iTOL[31] v.6 (https://itol.embl.de/)."*

3. The authors mention that the finding of multiple 7PET strains circulating in the same location is unusual, as pointed out in the manuscript. A recent study in Malawi found both ST69 and non-ST69 during the 2022–2023 outbreak in Malawi (https://www.medrxiv.org/content/10.1101/2023.06.08.23291055v1). Could you also comment in the introduction or discussion whether outbreaks caused by a single 7PET and non-7PET strains are also uncommon?

Authors: We now discuss the possibility of identifying 7PET and non-7PET isolates during a same outbreak in the discussion section. The text now reads as follows: *"The intensive use of culture media for the isolation of Vibrio species (such as TCBS) during cholera outbreaks can lead to the isolation of a non-pathogenic Vibrio spp. from a patient with diarrhoea not due to cholera. Lassalle and coworkers[6] sequenced 5.7% (250/4,375) of the V. cholerae O1 isolates recovered from suspected cholera patients in Yemen between 2018 and 2019 and found that 8% (n = 21) of these isolates were not toxigenic and did not belong to the 7PET lineage. The proportion of non-7PET isolates reached 30% (3/10) for their environmental isolates. However, only two of these 24 non-7PET isolates — from clades VcK and ST170 — were assigned to serogroup O1; the others belonged to other O groups (O7, O34, O146, O167). Here, we include only V. cholerae isolates clearly demonstrated to belong to the O1 serogroup. Furthermore, a random selection of 60 non-sequenced isolates were shown to contain the ctx gene. However, the presence of the ctx gene in V. cholerae O1 is not a 100% reliable indicator of membership of the 7PET lineage because this feature is also observed in other V. cholerae O1 lineages, such as L3, which includes the US Gulf Coast clone [14,16]. These non-7PET lineages are associated with sporadic seafood-borne infections but not epidemic cholera[14]. Based on our data and the epidemiological context of this explosive but short-lived cholera outbreak in Lebanon, we are confident that only a very small proportion of the 637 non-sequenced isolates were not the true toxigenic agent of cholera."* However, we have used the Yemeni context (rather than the Malawian context as suggested) to discuss the distribution of 7PET and non-7PET isolates, because Yemen is geographically closer to Lebanon and the paper by Lassalle et al was peer-reviewed and has been published (Nat. Microbiol 2023), and we therefore consider this citation preferable to that of a non-peer-reviewed preprint.

4. Consider discussing the introduction times of the two 7PET strains identified in Lebanon. For example, is it likely that the strains were introduced around the same time? If one of the strains was introduced much earlier than the other, the authors should speculate why it did not cause any outbreak before the 2022–2023 outbreak.

Authors: We have addressed this issue by indicating the introduction times of the AMR and XDR strains in the text (lines 112-117) and by adding the introduction dates of both strains to the revised Figure 1B. The first XDR isolate was isolated in October 22nd, 19 days after the isolation of the AMR strain (October 3rd). Of the two hypotheses initially considered (lines 117-122), this particular chronology led us to favour the hypothetical acquisition of an MDR plasmid by the strain with a narrower AMR profile initially responsible for the outbreak. This is discussed in the revised MS (Lines 202-214). However, this hypothesis was ruled out by the genomic analysis showing the introduction of two different 7PET strains (AFR15 or AFR13).

5. Some discussion focusing on the differences in the prevalence of the two strains would be great. Did the predominant strain harbour unique genomic features that would make it more virulent and transmissible than the minority strain? Or was the observed number of cases primarily due to the differences in the affected populations in the South of Lebanon and elsewhere?

Authors: This is a very sensitive issue in the political context of Lebanon and the current geopolitical context of the entire Middle East. We have therefore added the following explan ation: *"The strain with the narrower AMR profile predominated in all affected regions of Leba non, including North Lebanon in particular, whereas the XDR strain was found only in South L ebanon and the*
Beqaa Valley. This more limited distribution of the XDR strain may be due to the movements of people between these parts of Lebanon and Yemen in the current geopolitical context."

Reviewer #2 (Remarks to the Author):

The authors have done a comprehensive genome-wide analysis of 34 isolates randomly sampled from 671 V. cholera isolates that caused outbreak after 30 years in Lebanase during October'2022 to January '2023. Since Lebanon is not endemic to cholera and the exclusivity of the V. cholera strains in this outbreak from their previous two episodes indicates travel associated transmission.
The very containment of the infection with vaccination highlights the interlinked immunity mediated protection over the transmission of V. cholera in this outbreak.
The study indicates the presence of ctxB7 allele and the characteristic tcpACIRS101and VSP-IIΔ in all the 34 WGS variants. These variants emerged with the seventh pandemic V. cholerae El Tor (7PET) at the third wave and is associated with high Cholera toxin synthesis and high intercontinental transmission potency.
We also appreciated the authors WGS and phylogeny based approach to unlock the independent introduction of the two variants, the low AMR and MDR strains attributed to this outbreak.
The manuscript is well drafted and appreciated, however we have the following concerns to be addressed before it is further processed.

The authors may justify for sampling a very size small size (34/671) from the laboratory confirmed cases for WGS analyses.

Authors: We now discuss this 5% sampling in the discussion section. The text now reads as follows: *"One limitation of this study is that only a small proportion (5%, 34/671) of the*

outbreak isolates were sequenced. We cannot, therefore, rule out the possibility that some of the 637 non-sequenced V. cholerae O1 isolates do not belong to the 7PET lineage, the only lineage currently causing epidemic cholera. The intensive use of culture media for the isolation of Vibrio species (such as TCBS) during cholera outbreaks can lead to the isolation of a non-pathogenic Vibrio spp. from a patient with diarrhoea not due to cholera. Lassalle and coworkers[6] sequenced 5.7% (250/4,375) of the V. cholerae O1 isolates recovered from suspected cholera patients in Yemen between 2018 and 2019 and found that 8% (n = 21) of these isolates were not toxigenic and did not belong to the 7PET lineage. The proportion of non-7PET isolates reached 30% (3/10) for their environmental isolates. However, only two of these 24 non-7PET isolates — from clades VcK and ST170 — were assigned to serogroup O1; the others belonged to other O groups (O7, O34, O146, O167). Here, we include only V. cholerae isolates clearly demonstrated to belong to the O1 serogroup. Furthermore, a random selection of 60 non-sequenced isolates were shown to contain the ctx gene. However, the presence of the ctx gene in V. cholerae O1 is not a 100% reliable indicator of membership of the 7PET lineage because this feature is also observed in other V. cholerae O1 lineages, such as L3, which includes the US Gulf Coast clone [14,16]. These non-7PET lineages are associated with sporadic seafood-borne infections but not epidemic cholera[14]. Based on our data and the epidemiological context of this explosive but short-lived cholera outbreak in Lebanon, we are confident that only a very small proportion of the 637 non-sequenced isolates were not the true toxigenic agent of cholera."

We also wonder whether the genomic analyses be extrapolated to categorize the remaining phenotypically categorized V. cholerae strains as all the 671 were well grouped into two categorized low AMR and extensive MDR strains.

Authors: As all of the isolates had one of the two described antimicrobial susceptibility testing (AST) profiles (AMR or XDR) (see results, lines 130-140) we can extrapolate the genomic analysis to all isolates based on their corresponding AST profiles.

The frequent usage of the terminology of narrow resistance and extended MDR in the manuscript is confusing. We thus suggest the authors to categorize the strains as either antimicrobial resistant (AMR) /multidrug resistant (MDR) and extensive drug resistant XDR as addressed universally throughout this manuscript for better clarity.
Subsequently, the authors may define the criteria for MDR/ extended MDR in the methodology.

Authors: In accordance with the reviewer's suggestion, we now use the terms "AMR" for the narrower antimicrobial resistance profile and "XDR" (extensively drug-resistant) for the second profile throughout the manuscript.

We suggest for time reconstruction from the patient meta data to reconfirm the independent introduction of two different strains in Lebanon as against the other hypothesize of convergent evolution of the MDR strain from the predominant narrow resistant strains.

Authors: We now indicate the introduction times of the AMR and XDR strains in the text (lines 112-117) and by adding the introduction dates of both strains to the revised Figure 1B.

The first XDR isolate was isolated in October 22nd, 19 days after the isolation of the AMR strain (October 3rd). Of the two hypotheses initially considered (lines 117-122), this particular chronology led us to favour the hypothetical acquisition of an MDR plasmid by the strain with a narrower AMR profile initially responsible for the outbreak. This is discussed in the revised MS (Lines 202-214). However, this hypothesis was ruled out by the genomic analysis showing the introduction of two different 7PET strains (AFR15 or AFR13).

Line 82: Does the second case connection with the first case?

Authors: Yes, the second case was a health worker involved in the medical care of the first case. This information has been added to the revised manuscript.

The authors may provide a geographical of Lebanon, to better understand the contents provided in "72-92"; especially the locations of South of Lebanon (Tyr) and Beqaa, from where the MDR strains were predominantly isolated. We also insist relevent correction in Fig.1 b.

Authors: The map has been redrawn to address the various points raised by this reviewer.

Fig. 1b: The authors mention that the last reported case were in January'2023(Line No. 94). The cumulative suspected cases in the figure indicates logarithmic increase against the subsequently decreasing confirmed cases. We suggest an explanation for this.

Authors: Thank you for pointing out this discrepancy. The graph has now been redrawn. The cumulative suspected cases line has been replaced by a plot of non-cumulative suspected cases and confirmed cases. This should provide a better comparison while avoiding the confusion caused by the continuous increase of the cumulative suspected cases graph against the decreasing number of confirmed cases. The risk of suspecting cholera in patients with similar symptoms increases during a cholera epidemic, potentially accounting for the increase in the number of suspected cases relative to confirmed cases at certain time points.

Details regarding how many out of the 671 isolates were sent to AUB and how many were stored at LMSE. (Line 146-147) is confusing.

Authors: This sentence has been modified and now reads: "In total, 671 clinical isolates of *V. cholerae* were identified, with 144 isolates from North Lebanon collected and stored in the "la Collection Microbiologique de l'Université Libanaise (CMUL)" at LMSE at the Lebanese University. The remaining 527 isolates were stored at AUB."

The authors may provide information on how was the O antigen determined phenotypically for the 671 so that the categorization of the O1 biotype was confirmed.

Authors: This information has been added to the Methods section ("*Agglutination was performed with specific antisera (V. cholerae O1 Inaba Monovalent Antiserum, V. cholerae O1 Ogawa Monovalent Antiserum, V. cholerae O1 Polyvalent (Inaba, Ogawa) Antiserum and V. cholerae O139 Antiserum (Mast Assure, Liverpool, UK)), for all 671 isolates*") and the Results section ("*Serotyping and antimicrobial drug susceptibility testing in the Lebanese*

laboratories (LMSE and AUB) identified two different profiles — one AMR and one XDR (Table 1 and Supplementary Data 1) — in the 671 Vibrio cholerae O1 isolates recovered during the outbreak").

Line 324: Focusing on sub-genomic regions like mobile elements to understand cholera's spread has made El Tor isolates from the 7th pandemic appear more diverse than they truly are, compromising accurate molecular epidemiological mapping. In this context, we appreciate the author's approach of using SNV in place of SNP (which requires at least 1% prevalence of the variant(s). However, we insist the authors provide details of the bioinformatics methodologies to evaluate SNV and as well as construction of the phylogenetic tree.

Authors: We have constructed a maximum likelihood phylogenetic tree with RAxML software, based on 10,647 chromosomal non-recombinogenic SNVs. These SNVs — now shown in Supplementary Data 3 — were identified with Snippy and Gubbins after the mapping of our genomic sequences onto the reference genome of *V. cholerae* O1 El Tor N16961. This approach was previously used to identify the different 7PET sublineages and to assess their geographic spread (Weill et al. Science 2017; Weill et al. Nature 2019, Oprea et al Nat Commun 2020; Mashe et al. N Engl J Med 2020,). Our data have been confirmed by other approaches and groups (Ekeng et al. Elife 2021, Taylor-Brown et al. Nat Commun 2023; Lassalle et al. Nat Microbiol 2023). The different versions of these software suites and the non-default parameters used were shown. We believe that all the necessary details for the replication of this work have now been provided.

Fig.3: We insist on including AFR15 in the color code. May clarify details on how both the outbreak groups were placed against the AFR13 and in parallel to the Middle East region. Line 365-367 conveys that Lebanon isolates belonged to AFR15 sublineage.

Authors: We now include in the phylogeny the six African isolates from the AFR15 sublineage published by Smith et al (Emerg Infect Dis 2023). This AFR15 sublineage is labelled on Fig. 3 and is discussed in the text.

Reviewers' Comments:

Reviewer #2:
Remarks to the Author:
It was a pleasure evaluating the revised manuscript entitled "Genomics reveals an unusual two-strain cholera outbreak in Lebanon, 2022-2023”. The authors have addressed and justified all the concerns that were raised. The manuscript demonstrates the potency of Genomic surveillance to decode the origins of the massive cholera outbreak in Lebanon. The methodologies are well detailed and the analyses, interpretation and conclusions drawn are mechanistic and appreciable. I hence recommend the article for its further processing and I wish the authors a good luck.