nature portfolio

Peer Review File

Longitudinal genomics reveals carbapenem-resistant Acinetobacter baumannii population changes with emergence of highly resistant ST164 clone

Corresponding Author: Professor Yunsong Yu

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Synopsis:

This was a follow-up cross-sectional observational study of Acinetobacter baumannii in a 28-bed ICU in Hangzhou, China. The research team employed a deep-sampling approach followed by Illumina whole genome sequencing of all isolates identified from ICU patients and the ICU environment, and long-read Nanopore sequencing of selected isolates. Following infection prevention and control (IPC) interventions, they investigated and compared the genetic structures and dynamics of the A. baumannii population to those from a previous surveillance study conducted in 2019 using a similar approach and timeframe in the same ICU. Finally, they described the global phylogenetic structure and geographical distribution of an emerging carbapenem-resistant ST164 lineage.

Strengths:
The strengths of this study lie in its dense and unbiased sampling approach of all ICU-admitted patients and the ICU environment, coupled with whole genome sequencing and Nanopore sequencing. Furthermore, their dataset was analyzed in the context of a previous dataset from 2019 in the same ICU, providing an opportunity to understand the impact of interventions on bacterial population changes and antimicrobial resistance determinants between 2019 and 2021.

For example, this study found changes in the carbapenem-resistant Acinetobacter baumannii (CRAB) population, with a reduction in GC2 and a sharp increase in the emerging ST164 lineage carrying both ndm and oxa23 genes between 2019 and 2021, responsible for the overall increase in carbapenem resistance. They demonstrated the global emergence of carbapenem resistance via multiple mechanisms in ST164 isolates, highlighting a global warning about this clone. Similar to their previous study, they found that CRAB introductions associated with patients and ICU spread are driving population diversification; furthermore, patients likely acquire A. baumannii in the ICU prior to infection development. While these findings are not novel compared to their previous publication in Lancet Regional Heath, they clearly demonstrate the need for improving IPC measures.

Limitations:
The IPC interventions did not occur immediately after the first study as planned, but were instead interrupted by the COVID-19 pandemic. Therefore, the impact of IPC interventions is hard to distinguish from the effects of COVID-19, which occurred in 2020, between their two study periods (August-October 2019 and May-July 2021). COVID-19 likely impacted the A. baumannii population changes in the ICU, considering the surge in cases, increased antibiotic use, and IPC measures. It is challenging to determine if the observed population changes are directly linked to the IPC interventions initiated in late 2020. The rise and spread of ST164 is an important finding. However, investigating its environmental spread and low prevalence as a cause of infection was not part of this study.

Remarks:
This study approach is not novel considering their previous publication in Lancet Regional Heath; however, the findings are helpful in understanding the factors driving the changing dynamics of the Acinetobacter baumannii population in the ICU. It also provides new insights into the emergence of the CRAB ST164 lineage, which warrants further investigation.

My specific questions and comments:
Title: “Globally-significant” seems overstated, given its low prevalence in causing infections compared to previously characterized clones. Suggest edit or discuss.

Introduction:

Line 59: Reference 1 does not support the narrative that CRAB can persist in patients for extended periods.
Line 69: What is the definition of “persistent populations”?
Line 71: Suggest using the term “nosocomial settings” or “hospital environments.”

Methods:

Line 115: The specific duration of IPC interventions should be clearly described.
Line 132: Suggest providing information about the process of microbiological diagnostics of A. baumannii infection in the study setting for the audience's benefit.
Line 195: Include the name and accession number of the reference genome. What is the size of the core genome alignment?
Line 197: How many polymorphic sites were outputted from Gubbins analysis?
Line 199: Did the authors test for a temporal signature (e.g., using TempEst or similar tools) before constructing the time tree in IQ-tree? If yes, please provide the supporting data.
Line 213: Suggest adding the total number of SNP sites of ST164 here. Should time tree analysis be conducted for the newly emerging clone ST164?

Results:

Line 226: Are there any differences in length of stay (LoS) or ICU stay between A. baumannii-infected patients compared to other ICU patients? This information should be included.
Line 238: Other sources of A. baumannii isolation from patients should be provided. Can the authors comment on evidence of persistent carriage in the oral cavity or gut of ICU patients?
Line 248: The authors compared the CRAB isolation rates between 2019 and 2021 in the discussion but did not provide the 2019 data in the results. Suggest adding it here.
Line 262: What evidence supports this? Did the authors use routine hospital microbiological culture results or just data from the two sampling timeframes? Did the authors have access to routine culture data? Suggest keeping this sentence only if available data supports it.
Line 269: There were 34 different STs in Fig 1A.

Figure 1: 1 CSAB should be 4 CSAB; suggest editing. The model of the changing population should be modified to reflect the true population changes of CRAB.

Line 276: IC11 is a suggestion rather than a declaration; suggest edit or discuss.
Line 277: The first citation of reference 22 is not suitable.
Line 278: It should read “213 GC2 isolates.”
Lines 315-338: The labeling of “diverse central cluster” and “more clonal clusters” was not visible on Fig 2A. The division of 2019 clusters (C1-C17) and 2021 clusters (C18-C25) implies they are different, but there seems some overlapping, causing confusion and making it hard to follow the narrative. The authors claimed, “GC2 population was more diverse in 2021 than it was in 2019, consistent with frequent and ongoing introductions of distinct GC clusters to the ICU.” What do you mean by more diverse? Does this figure support such a claim?

The labeling of transient GC2 clusters throughout Fig 2A-2C needs to be clearly defined and presented. It was difficult to follow the narrative. For example, in lines 330 and 334, the sentences are difficult to understand. The number of patient samples within each transient cluster needs to be included.

Figure 2A: The labels of clusters, AMR genes, and plasmid replicons are very small. Suggest improving it.

Line 342: What do you mean by “study 1”?

Line 351: There should be a paragraph describing the sources of isolation for ST164 isolates, including types of positive samples from patients and environment? This information will be beneficial for future interventions.

Line 380: Is there a confidence interval for the estimated divergence dates from the time tree? Suggest providing SNP separation onto the time tree to indicate how many SNPs separate the four ST164 clusters.

Line 387: What does “an existing ICU population” mean?

Line 393: Suggest editing the sentence to improve clarity.

Line 395: What does “persistent ICU population” mean?

Line 414: The sentence is not well supported by figure 2. Suggest improving labeling to enhance clarity.

Line 414: “acquisition elsewhere.” The authors should provide info about the sources of A. baumannii-infected patients (direct admission or transfer) to support the sentence and discussion point (line 521).

Line 425: What data supports this claim?

Line 428: The sentence is unclear. What does it mean?

Line 439: Figure 5B should be placed separately from Fig 5A to enhance visualization.

Line 441: Data regarding the distinctions of KL types between GC2 and ST164 isolates should also be provided in the manuscript.

Line 443: What criteria were used for the designation of 7 clades?

Line 448: Does clade 2 include all the ST164 isolates from this study?

Line 449: Information about other clades and their CR genes need to be included.

Lines 454-482: The authors provided a model for the evolution of ST164 towards carbapenem resistance; however, many assumptions in their model are not supported by data (line 461, line 465, line 467). Suggest removing this section or analyzing the evolutionary pathways in the context of the phylogenetic tree and genomic evidence, focusing on phylogenetic branches with sufficient evidence.

Line 474: Figure 5B does not support the sentence.

Line 523: Can the authors provide further clarifications on why this method will prevent CRAB imports, given their available data? Any further suggestions?

Line 528: Suggest rephrasing “diversifying in situ” to improve clarity for the readers.

Line 538: Do the authors have data from other hospitals in China about this ST164 to support the sentence?

Line 551: I don’t think the study data supports this claim. Suggest to edit.

Discussion:

Line 509: The impact of IPC interventions could not be established between two studies conducted in 2019 and 2021 with a long delay in between due to COVID-19. The authors should include a limitation paragraph to discuss these issues.
Line 516: Suggest rephrasing the sentences to improve their meaning.

Conclusion:
There should be a conclusion paragraph to recapitulate the study, its finding and its implication.

Reviewer #2

(Remarks to the Author)
Large number of genomes analysed

The manuscript by Yu and colleagues describes the emergence of a globally-significant A. baumannii ST164 clone in a Hangzhou ICU. The study uses surveillance isolates and a comprehensive genomics pipeline to examine isolates recovered from two separate longitudinal studies. When combined with a large number of publicly-available genomes, the analysis identifies a significant shift in the clonality of important carbapenem-resistant isolates in the hospital setting. The data also reflects an increase in carbapenem resistance levels in the ICU between the first and second studies, and ultimately shows a bi-clonal (ST2 and ST164) population of carbapenem resistant isolates. The study also identifies multiple introductions of GC2 into this hospital setting.

This body of work is comprehensive, highly relevant, and well-written. It leverages up-to-date genomics pipelines to process and analyse an impressive number of genomes, and the analysis is sound. The authors are also commended for having all of the genomics data, including assemblies and reads, available for review in the BioProject.

My only comment relates to the figures. The figures are nicely presented, but are let down by the size of the labelling. Particularly for the phylogenetic trees, the column labels are essentially unreadable because of how small they are, and as a result the reader gets lost in trying to work out what is being shown. Improvements to the size and clarity of the labelling would significantly improve the figures and the reading experience for the reader.

Reviewer #3

(Remarks to the Author)
The manuscript "Longitudinal genomics reveals changes to carbapenem-resistant Acinetobacter baumannii population with the emergence of a globally significant ST164 clone" draws an interesting genomic picture of the globally significant ST164 A. baumannii strains. It is a well-written manuscript and includes much data and analysis. I only have a few general and specific comments as listed below:

- Line 63: The latest update was in April 2024, please use this: https://www.who.int/publications/i/item/9789240093461.

- Line 267: Please include the total number "All (n=?)".

- Please replace "pDETAB10::Tn2006" with "pDETAB10/Tn2006" as "::" often indicates insertion/interruption of an element/chromosome/gene/etc.

- Line 311: Was this the case (Re acquisition of Tn2006/Tn2009 via homologous recombination)?

- not sure if the "top part of Panel A in Figure 1" is needed, it is too basic and does not add value. It takes away the attention from the more important parts of this Figure with more meaningful info.

- Line 364: It would be interesting to provide more info on the chromosomal location and genetic context of Tn6924 and whether that is possible that this Tn might have been acquired by homologous recombination.

- Line 367: Were Tn2006 and Tn6168 in novel chromosomal positions. This can potentially reveal links to other sequence types with the same transposons. This comment involves Lines 461 and 470-471.

- Line 441: Was KL47 also tracked? Can you please include more info on other STs (from the literature) that include KL47? This would potentially reveal the interaction of their common ancestors.

- line 461: What about in publicly available genomes?

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have addressed the comments and questions raised in my initial review, and their responses have clarified key points in the manuscript. The revisions, both in the content and structure, have significantly improved the overall clarity and presentation of the work. I appreciate your efforts in thoroughly addressing the feedback.

REVIEWER COMMENTS

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isolates, highlighting a global warning about this clone. Similar to their previous study, they found that CRAB introductions associated with patients and ICU spread are driving population diversification; furthermore, patients likely acquire A. baumannii in the ICU prior to infection development. While these findings are not novel compared to their previous publication in Lancet Regional Heath, they clearly demonstrate the need for improving IPC measures.

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This study approach is not novel considering their previous publication in Lancet Regional Heath; however, the findings are helpful in understanding the factors driving the changing dynamics of the Acinetobacter baumannii population in the ICU. It also provides new insights into the emergence of the CRAB ST164 lineage, which warrants further investigation.

**Thank you for your advice. We appreciate your comments and suggestions which are valuable for improving our paper and helpful in promoting the importance of our work. According to the comments and suggestions, a word-by-word revision has been made on the manuscript and amendments are highlighted in the revised version.

My specific questions and comments:

Title: “Globally-significant” seems overstated, given its low prevalence in causing infections compared to previously characterized clones. Suggest edit or discuss.

**We have changed the title to reflect this. The title is now: Longitudinal genomics reveals changes to a carbapenem-resistant *Acinetobacter baumannii* population with emergence of a highly resistant ST164 clone.

Introduction:

Line 59: Reference 1 does not support the narrative that CRAB can persist in patients for extended periods.

**Thanks for your careful review. We found this paper only showed *Acinetobacter* spp. has a significant capacity for long-term survival in the hospital environment. Hence, we have removed “and patients” from this sentence in the manuscript.

Line 69: What is the definition of “persistent populations”?

**In the publication of “Repeated local emergence of carbapenem-resistant *Acinetobacter baumannii* in a single hospital ward”, the study from Mark B. Schultz *et al*. (ref. 9) showed two novel clades were successful in persisting within the local population for several years. We would like to consider this kind of bacterial population is “persistent populations”.

Line 71: Suggest using the term “nosocomial settings” or “hospital environments.”

**We have revised the term to “nosocomial settings”.

Methods:

Line 115: The specific duration of IPC interventions should be clearly described.

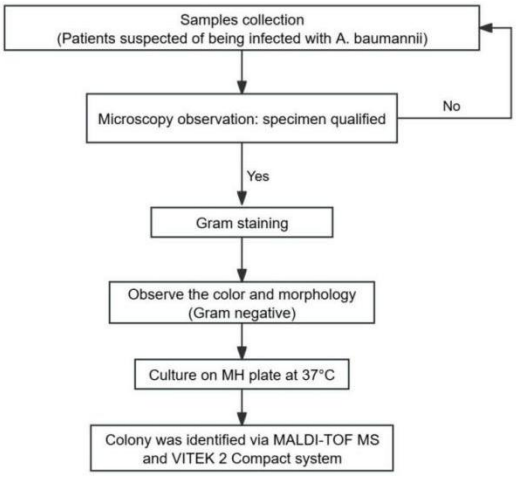
**The specific duration of IPC interventions was from September 2020 to July 2021. “September 2020” is the time of 8 months prior to the outset of this study (May 2020).

We have added the specific duration in the manuscript. Please see line 118.

Line 118: A bundled set of interventions (from September 2020 to July 2021) that aimed to address the abundance of CRAB in this ICU were implemented 8 months prior to the outset of this study.

Line 132: Suggest providing information about the process of microbiological diagnostics of *A. baumannii* infection in the study setting for the audience's benefit.

**Thanks for your comments. Clinical specimens (e.g., sputum, urine, or other appropriate body fluids) were collected from patients with suspected *A. baumannii* infections (based on clinical symptoms such as fever and cough). After sample collection, microscopy was used to check whether the quality of specimen is qualified (e.g., sputum: the number of white blood cells under low power microscope is greater than 25, the number of squamous cells is less than 10, and the ratio of squamous cells to white blood cells is less than 1:2.5) and further observe the presence of bacteria and their color, morphology based on the Gram staining. The sample was further cultured using MH agar plates at 37°C. Finally, the colony was identified using Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) (bioMérieux, France) and the VITEK 2 Compact system. We have added a flow chart (new Fig S2) in the supplementary figures file of the process of microbiological diagnostics.



Line 195: Include the name and accession number of the reference genome. What is the size of the core genome alignment?

**The reference genome was *A. baumannii* DETAB-R21 (GenBank accession GCA_024205285.1). The size of the core genome used in the alignment was 3,709,099 bp. We have added these data in line 200-201.

Line 197: How many polymorphic sites were outputted from Gubbins analysis?

**120 polymorphic sites. We have added it in the manuscript (line 202).

Line 199: Did the authors test for a temporal signature (e.g., using TempEst or similar tools) before constructing the time tree in IQ-tree? If yes, please provide the supporting data.

**Thanks for your question. We did not test for a temporal signature using TempEst for the IQ-tree. The dates were obtained by adding dates of isolation to the IQ-TREE command (http://www.iqtree.org/doc/Dating), which we note in the methods.

Line 213: Suggest adding the total number of SNP sites of ST164 here. Should time tree analysis be conducted for the newly emerging clone ST164?

**The total SNP number among 135 ST164 strains was 3-6037 (Table S6). We have added this number in the manuscript (line 219).

As for the time tree analysis for the global ST164 clone, we try to do that according to your advice. However, we tried to do that for many times using different parameters and we found that some Effective Sample Size (ESS) values are still less than 200. The specific details are as follows, please see below:

We firstly performed root-to-tip regression analysis using TempEst version 1.5.3 (https://github.com/beastdev/Tempest) and found the R^2 is 1. We then used a Bayesian Markov Chain Monte Carlo (MCMC) approach implemented in BEAST v1.10.4 with XML files generated with BEAUti v1.10.4 to analyze alignment of putative

substitution mutations identified by Gubbins. We allowed each run 30 million iterations, sampled from every 20,000th iteration, and discarded the first 10% of the samples as burn-in. MCMC chain was inspected in Tracer v1.7.2, with all parameter effective sampling sizes being above 200. The maximum clade credibility (MCC) tree was calculated using TreeAnnotator v1.10.4, which was plotted in FigTree v1.4.4. The time tree was shown as below. However, some ESS values are less than 200. The website recommends the ESS > 200 would be better (https://beast.community/ess_tutorial).

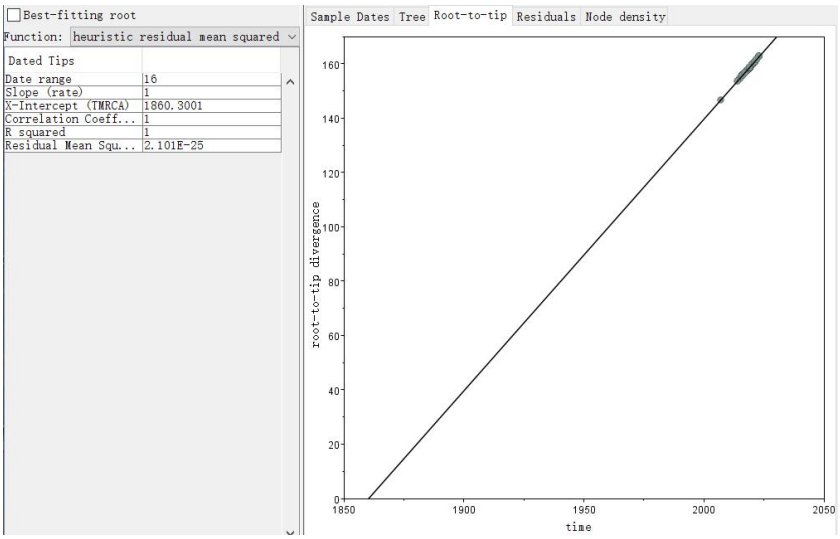


Fig. 1. root-to-tip regression analysis

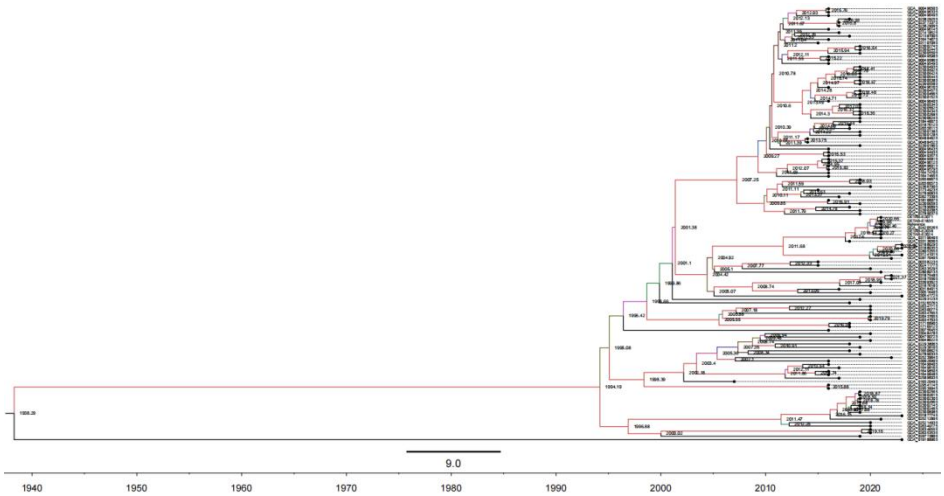


Fig. 2. A time-calibrated phylogeny of ST164 clone

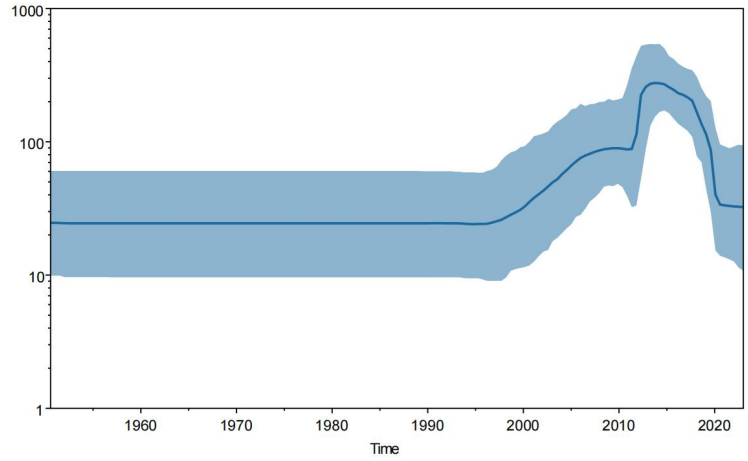


Fig. 3. Bayesian skyline plot

We further allowed each run 200 million iterations using strict clock model, sampled from every 20,000th iteration, and discarded the first 10% of the samples as burn-in. However, results showed some ESS values are still less than 200 (Fig. 4). Thus, we think the time tree analysis results were not good due to the inappropriate ESS values and we did not add this section into the manuscript. Thanks for your good suggestion.

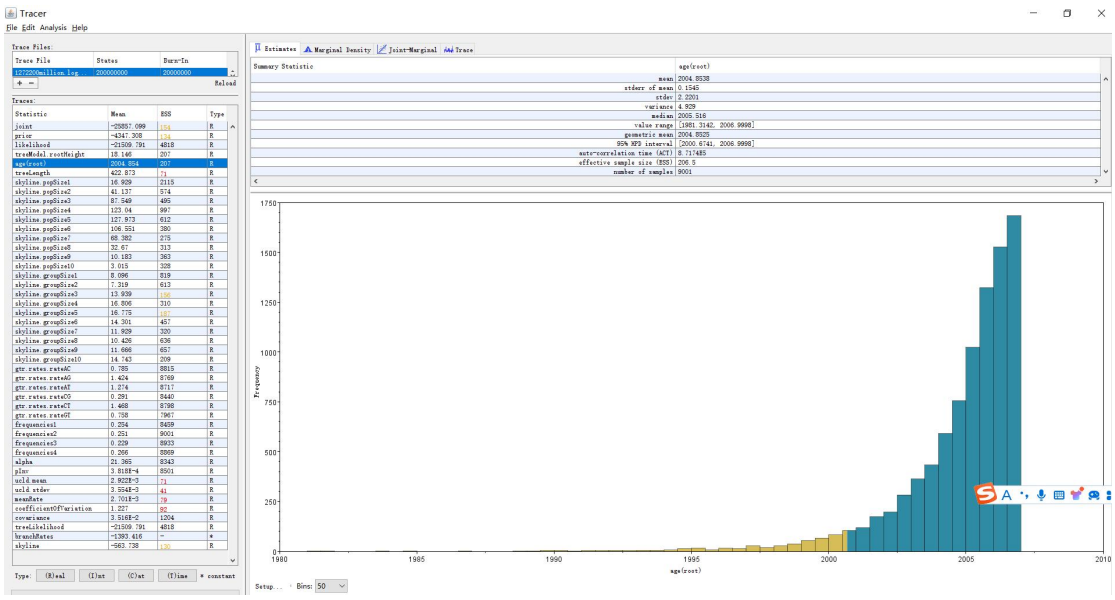


Fig. 4. ESS analysis using Tracer

Results:

Line 226: Are there any differences in length of stay (LoS) or ICU stay between A.

baumannii-infected patients compared to other ICU patients? This information should be included.

**Thanks for the question. We have performed the analysis of this. Results showed the median length of ICU stay of *A. baumannii*-infected patients (clinical isolative positive) was 28.5 days (IQR: 24 - 42.3 days). The median length of other ICU patients was 9 days (IQR: 5 - 16 days). The length of stay (LoS) of A. baumannii-infected patients was significantly longer than other patients in the ICU. We have added the related content in the manuscript. Please see line 232-235.

Line 238: Other sources of A. baumannii isolation from patients should be provided. Can the authors comment on evidence of persistent carriage in the oral cavity or gut of ICU patients?

**We have added the other sources of *A. baumannii* isolation from patients. Please see "Of the 131 patients screened, 41.2% (54/131) were *A. baumannii*-positive on at least one sampling occasion, with most of the positive samples originating from nasogastric tube (33/174, 19.0%) or oral (40/220, 18.2%) swabs, followed by rectal swabs (38/273, 13.9%), nasojejunal tube (4/49, 8.2%), endotracheal tube (5/94, 5.3%) and tracheostomy tube (3/81, 3.7%)." in line 247-251.

A. baumannii could persist in the oral cavity based on the positive samples of oral swabs in ICU patients. Our previous research showed *A. baumannii* DETAB-R21 was isolated from oral swab in the ICU[1], which was considering as one colonization isolate. Moreover, a study from A M Richards *et al*. showed subgingival colonization by *A. baumannii* could increase the risk of refractory periodontitis[2]. Apart from oral colonization, a study from Patrick M Ketter *et al*. revealed the gastrointestinal (GI) tract has been found to be a major reservoir for *A. baumannii*, as well as to potentially contribute to development of multidrug resistance[3]. Our study also showed that nasogastric tubes have a high CRAB isolation rate. These persistent carriage strains in patients require further attention in the ICU. We have added these data in the discussion section. You could kindly find them in line 513-525.

Line 248: The authors compared the CRAB isolation rates between 2019 and 2021 in the discussion but did not provide the 2019 data in the results. Suggest adding it here.

**We have added this information in the results section. Please kindly find "However, the isolation rate of CRAB clinical strains of patients was decreased from 8.6% (2019) to 7.6% (2021)." in line 260-261.

Line 262: What evidence supports this? Did the authors use routine hospital microbiological culture results or just data from the two sampling timeframes? Did the authors have access to routine culture data? Suggest keeping this sentence only if available data supports it.

**Thanks for your question. The evidence supporting this is derived from the MIC tests performed as part of this study. We have the routine hospital microbiological culture results and have included these isolates, namely DETAB-C1 to C19 from the 2019 study and DETAB-D1 to D13 from this study. Routine hospital microbiological culture results have therefore already been integrated into the two sampling timeframes. Altogether, we observed an overall increase in carbapenem resistance in this ICU between 2019 and 2021.

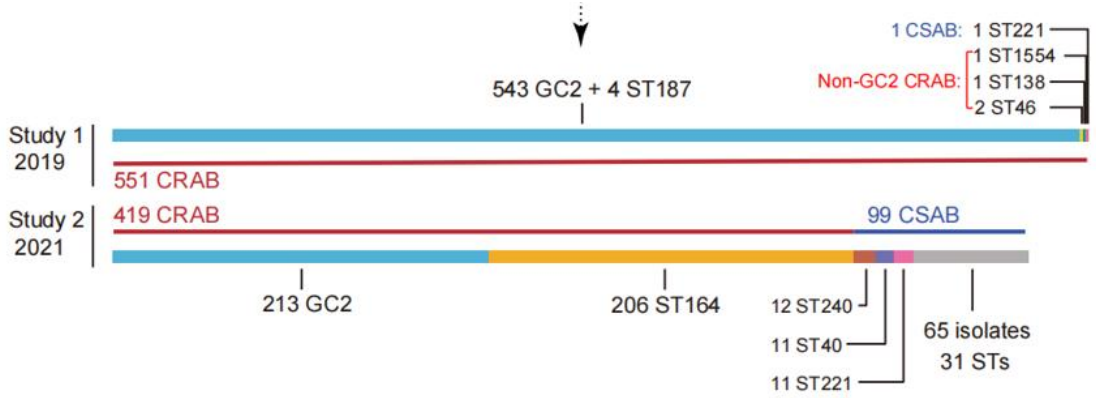
Line 269: There were 34 different STs in Fig 1A.

**Thanks for your careful review. We have revised the "33" to "34". In addition, we found in the original "Genome sequence data revealed that the population included representatives of 35 different sequence types according to the Pasteur MLST scheme (Fig. 1A)." The "35" should be "36". We have modified it in the manuscript (line 280).

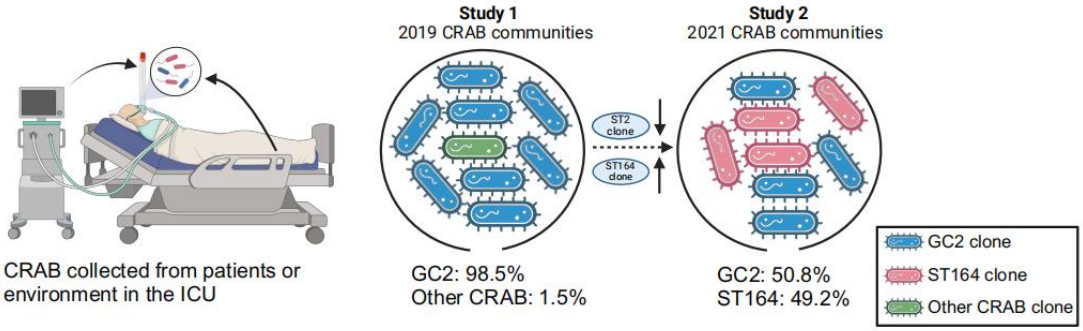
Figure 1: 1 CSAB should be 4 CSAB; suggest editing. The model of the changing population should be modified to reflect the true population changes of CRAB.

**Thanks for your careful review. In the 2019 study, 1 CSAB is right. However, there are 4 non-GC2 CRAB (1 ST1554, 1 ST138 and 2 ST46) below the 1 CSAB (1

ST221), and this maybe caused some confusion. We have revised the figure (labelling the non-GC2 CRAB) for clarity. Please see the new Fig. 1A.



As for the model of Fig. 1A, we have revised the Fig. 1A (please see below) to reflect the true population changes of CRAB with their isolation rate based on the real data in this study. This figure may be able to more vividly reflect the true population changes of CRAB.



Line 276: IC11 is a suggestion rather than a declaration; suggest edit or discuss.

**We have modified this sentence to "ST164 isolates were obtained from clinical specimens in this ICU earlier in 2021, and this ST has recently been described as a new international clone (IC11), with reports of human infections in Europe, Asia, Africa and the Middle East." Please kindly find it in line 288-291.

Line 277: The first citation of reference 22 is not suitable.

**Thanks for your kind reminder. We found that it is repeated in reference 22 at the end of the sentence. So, we have removed the first reference 22 in this sentence.

Line 278: It should read “213 GC2 isolates.”

**Has been revised.

Lines 315-338: The labeling of “diverse central cluster” and “more clonal clusters” was not visible on Fig 2A. The division of 2019 clusters (C1-C17) and 2021 clusters (C18-C25) implies they are different, but there seems some overlapping, causing confusion and making it hard to follow the narrative. The authors claimed, “GC2 population was more diverse in 2021 than it was in 2019, consistent with frequent and ongoing introductions of distinct GC clusters to the ICU.” What do you mean by more diverse? Does this figure support such a claim?

** To better reference parts of the figure in the manuscript text, we have labelled parts of the phylogeny “x”, “y”, or “z” to more explicitly highlight the clusters we refer to in our descriptions of the population. Words have been added to the beginning of this results section (lines 326-328) outlining the overall structure of the phylogeny, with reference to the new labels for clarity. Regarding diversity: horizontal distances in phylogenies like the one in Figure A correspond to relationship distances, with longer horizontal lines indicative of greater differences between nodes. The appearance of an entirely new, and distantly related node in 2021 (z), and the appearance of additional sub-clusters within the most diverse cluster (x) are consistent with our statement. We have further emphasized this in the manuscript text. We hope that our changes to Figure 2 and our initial description of the phylogeny will further improve the clarity of our presentation.

Within Figure 2, we have modified the following: increased text sizes for all labels, added additional labels to parts of the phylogeny (x, y, z), expanded the 2021 cluster column to the right of the phylogeny for emphasis, added the words “transient clusters” to our label for part C, and repeated the color key for transient clusters in part C.

The labeling of transient GC2 clusters throughout Fig 2A-2C needs to be clearly

defined and presented. It was difficult to follow the narrative. For example, in lines 330 and 334, the sentences are difficult to understand. The number of patient samples within each transient cluster needs to be included.

**We have addressed this as outlined in our answer above. Additional labels for these transient clusters have been added to Fig. 2C, re-emphasizing the color key that continues from Fig. 1A and Fig. 1B. We have also further emphasized which clusters were transient in-text.

As for the number of patient samples within each transient cluster, we have added these numbers in the manuscript. Please see "The numbers of patient samples within each transient cluster (C18-C23) were 1, 5, 1 ,7, 1 and 2, respectively." in line 344-346. We have also labelled these data in the Fig. 2C.

Figure 2A: The labels of clusters, AMR genes, and plasmid replicons are very small. Suggest improving it.

**The sizes of all labels in the Fig. 2A have been enlarged, and further labels have been added as described in our answers above.

Line 342: What do you mean by "study 1"?

**Study 1" refers to our previous study in this ICU in 2019. To clarify, we have replaced "study 1" here with "the 2019 study".

Line 351: There should be a paragraph describing the sources of isolation for ST164 isolates, including types of positive samples from patients and environment? This information will be beneficial for future interventions.

**Thanks for your good suggestion. We have added a paragraph describing these. Please kindly find "Over the course of this study, we obtained 206 ST164 isolates from 26 different sample types from patients and the ICU environment. The three most common positive sample types were oral (17/206, 8.3%), nasogastric tube (12/206, 5.8%) and rectal (12/206, 5.8%) swabs for patients, and bedside table

(25/206, 12.1%), ventilator (22/206, 10.7%) and bed rail (16/206, 7.8%) swabs for the environment." in line 368-373.

Line 380: Is there a confidence interval for the estimated divergence dates from the time tree? Suggest providing SNP separation onto the time tree to indicate how many SNPs separate the four ST164 clusters.

**Thanks for the question. There is not. The SNP distances between the four ST164 clusters are now indicated in the figure (Fig. 3B).

Line 387: What does "an existing ICU population" mean?

**To clarify, we have modified the sentence to appear as follows: "emergence from a pre-existing ICU population rather than from patient-associated introductions over the course of this study."

Line 393: Suggest editing the sentence to improve clarity.

**We have changed this to "All of these patient-derived ST164 isolates were identical (0 cgSNPs; Table S4) to one or more isolates that had previously been obtained from the ICU environment or from other ICU patients." to improve its clarity.

Line 395: What does "persistent ICU population" mean?

**We have modified this to "the population persisting in the ICU" for clarity.

Line 414: The sentence is not well supported by figure 2. Suggest improving labeling to enhance clarity.

**Labels in Fig. 2 have been as outlined in the comments above.

Line 414: "acquisition elsewhere." The authors should provide info about the sources of *A. baumannii*-infected patients (direct admission or transfer) to support the sentence and discussion point (line 521).

**Thanks for your comment. We have the clinical data of *A. baumannii*-infected

patients and data showed that 13 clinical CRAB isolates were collected from 10 *A. baumannii*-infected patients. Among them, 5 patients were admitted directly to the ICU (from different hospitals or the community), and another 5 patients were transferred from other wards in the hospital. P200 is one patient who was admitted directly to the ICU. However, we do not know whether they had spent time in a different hospital prior to admission. Therefore, the C21 cluster was introduced to the ICU from elsewhere (outside of Sir Run Run Shaw hospital). According to the supporting information, we have added to the sentence. Please see "The GC2 C21 cluster appears to have been introduced to the ICU by patient 200 (P200) (see above), who was admitted to the ICU from either the community or a different hospital (data not available). The presence of C21 in a P200 clinical sample is therefore indicative of acquisition outside this hospital, before the development of clinically-relevant symptoms requiring sputum sample collection in this ICU." on line 436-440.

Line 425: What data supports this claim?

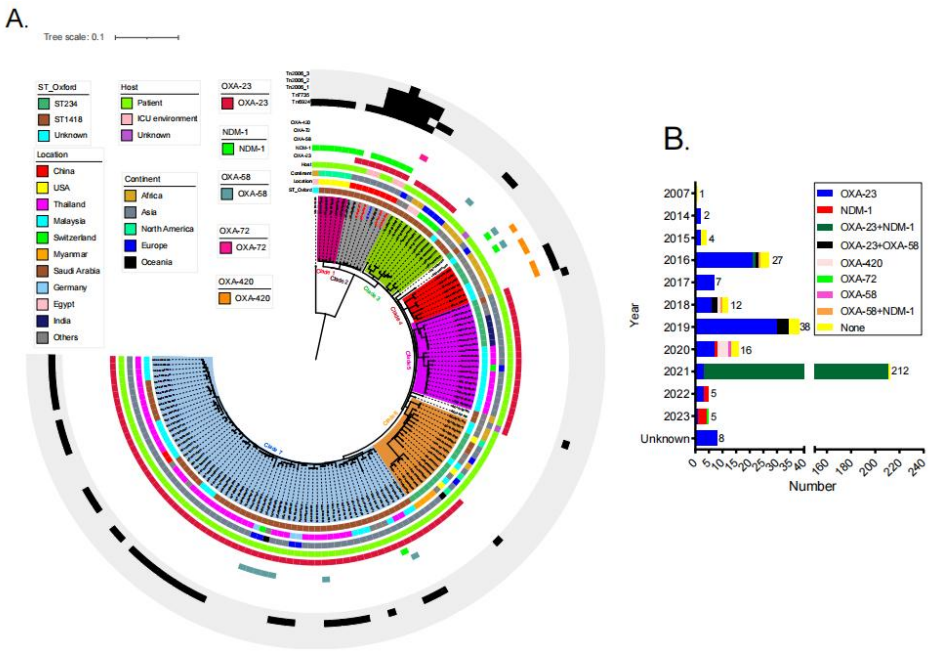
**Our core-gene nucleotide identity (cgSNP) data supports this claim. To clarify that, we have revised the sentence as follows: "In three of these cases isolates identical to the clinical isolates (0 cgSNPs) had previously been isolated from the ICU environment. It therefore seems most parsimonious that these patients acquired CRAB in the ICU."

Line 428: The sentence is unclear. What does it mean?

**We have clarified the sentence as follows: "The five remaining clinical isolates were not identical to any other isolates in the collection, but differed from multiple isolates by ⩽4 cgSNPs."

Line 439: Figure 5B should be placed separately from Fig 5A to enhance visualization.

**Thanks for your advice. Fig. 5 has been revised to enhance visualization according to your suggestion. Please see below:



Line 441: Data regarding the distinctions of KL types between GC2 and ST164 isolates should also be provided in the manuscript.

**Thanks for your advice. We have further analysed the KL types of 213 GC2 isolates. Results showed the most common KL types were KL30 (43.2%, 92/213) and KL93 (33.8%, 72/213). We have revised the sentence to “The most common KL type in global ST164 isolates was KL47 (63.4%, 83/131), which differs from the GC2 isolates where KL30 (43.2%, 92/213) and KL93 (33.8%, 72/213) were most common.” Please kindly find them in line 463-465.

Line 443: What criteria were used for the designation of 7 clades?

**Thanks for your question. Initially, we aimed to define the different clusters using fastbaps. We analysed the 135 ST164 strains using the fastbaps level 7 parameter and results showed that 115 strains belonged to a single clade. However, some strains within this fastbaps cluster were located on clearly distinct branches of the phylogenetic tree. So, in order to better describe the characteristics of these isolates, we divided them into seven main clades with the direct support of phylogenetic branches.

Line 448: Does clade 2 include all the ST164 isolates from this study?

**Thanks for your comment. It encompasses the breadth of diversity in this study's ST164 population. Our 206 ST164 isolates could be divided into 4 clusters (Fig. 3). In order to eliminate the noise of saturating a phylogeny with 206 very closely related (0-23 cgSNP) ST164 strains on the global evolutionary tree, we chose DETAB-E1835, DETAB-E2024, DETAB-E2038 and DETAB-E2071 (one from each cluster) to represent the complete diversity of ST164 isolates from this study. All four of these isolates were found in clade 2 of the global phylogeny, as would all ST164 isolates from this study.

Line 449: Information about other clades and their CR genes need to be included.

**Thanks for your suggestions. We have added this information. Please kindly find "Isolates of other clades (clade 1, clade 3-7) carry at most one carbapenem resistance gene. Moreover, all strains from clade 1 carry *bla*$_{\text{NDM-1}}$ and the majority of clade 3-7 isolates only harbored *bla*$_{\text{OXA-23}}$." in line 475-477.

Lines 454-482: The authors provided a model for the evolution of ST164 towards carbapenem resistance; however, many assumptions in their model are not supported by data (line 461, line 465, line 467). Suggest removing this section or analyzing the evolutionary pathways in the context of the phylogenetic tree and genomic evidence, focusing on phylogenetic branches with sufficient evidence.

**This section has been removed from the manuscript now.

Line 474: Figure 5B does not support the sentence.

**Thanks for your kind reminder. It should be "Fig. 6G". We have revised it, but this section has been removed now.

Line 523: Can the authors provide further clarifications on why this method will prevent CRAB imports, given their available data? Any further suggestions?

**Thanks for your comment. We reread this sentence and found it is a bit

inappropriate for the description of “address CRAB import”. What we would like to express is the mean of “prevent CRAB spread in the ICU”. We have modified the “address CRAB import” to “prevent CRAB spread in the ICU” in this sentence.

As for other suggestions, we think if patients are screened for CRAB prior to admission, carriers can be admitted directly to an isolation ward and cared for by a single, dedicated nurse to prevent the introduction and dissemination of CRAB in this ICU. We have added related description “To further prevent its dissemination in this ICU, the patients can be admitted directly to the isolation ward and cared for by only one nurse if the screening result is positive before the patients prior to the ICU admission” in line 531-533.

Line 528: Suggest rephrasing “diversifying in situ” to improve clarity for the readers.
**This has been changed to “diversifying in the ICU”.

Line 538: Do the authors have data from other hospitals in China about this ST164 to support the sentence?
**Thanks for your question. Until now, only in Hangzhou, we found the ST164 strains carried *bla*$_{\text{NDM-1}}$ and *bla*$_{\text{OXA-23}}$ simultaneously. Hence, we revised the original sentence from “Chinese hospital settings” to “our hospital settings”.

Line 551: I don’t think the study data supports this claim. Suggest to edit.
**Thanks for your comments. We agree with your point of view. Our study did not address virulence, and this sentence is inappropriate. Hence, we have removed this sentence from the manuscript.

Discussion:

Line 509: The impact of IPC interventions could not be established between two studies conducted in 2019 and 2021 with a long delay in between due to COVID-19.

The authors should include a limitation paragraph to discuss these issues.

**Thanks for your comments. We agree with your suggestions and we have added one paragraph to describe the limitation. Please see below in 571-577.

Line 571-577: A limitation of this study is that the IPC interventions did not occur immediately after the first study in 2019 due to the COVID-19 pandemic. COVID-19 may have played a role CRAB population changes in the ICU, considering the surge in cases, increased antibiotic use, and associated IPC measures. Hence, it is difficult to accurately measure the impact of IPC interventions and COVID-19 on the ICU CRAB population. We expect that the CRAB population was influenced by the combined effects of both our IPC interventions and COVID-19-related changes to clinical practice.

Line 516: Suggest rephrasing the sentences to improve their meaning.

**We have modified the sentences as follows: We used a highly-stringent 0-cgSNP cut-off for assessing putative transmission events over the course of this study. This allowed us to unambiguously implicate environmental persistence in the development of hospital-acquired infections. Please kindly find them in line 523-525.

Conclusion:

There should be a conclusion paragraph to recapitulate the study, its finding and its implication.

**Thanks for your suggestion. We have added a conclusion paragraph. Please see below in 579-587.

Line 579-587: In conclusion, we report on the emergence of a new CRAB clone, ST164, in this Hangzhou ICU. Our data show that ST164 was as successful as the globally dominant clone GC2 in this ICU setting, but exhibited higher carbapenem resistance levels due to dual production of NDM-1 and OXA-23 carbapenemases. Through comparative analyses, we found that ST164 has been isolated in 26 countries

from five continents, and has evolved towards carbapenem resistance on multiple independent occasions. We conclude that ST164 is an emerging high-risk lineage, which requires urgent global surveillance and our findings will lay a foundation for the formulation of more reasonable and effective targeted IPC strategies for ICU staffs, patients and environment.

We appreciate for your warm work earnestly, and hope that the correction will meet with approval. Once again, thank you very much for your comments and suggestions.

Reviewer #2 (Remarks to the Author):

Large number of genomes analysed

The manuscript by Yu and colleagues describes the emergence of a globally-significant A. baumannii ST164 clone in a Hangzhou ICU. The study uses surveillance isolates and a comprehensive genomics pipeline to examine isolates recovered from two separate longitudinal studies. When combined with a large number of publicly-available genomes, the analysis identifies a significant shift in the clonality of important carbapenem-resistant isolates in the hospital setting. The data also reflects an increase in carbapenem resistance levels in the ICU between the first and second studies, and ultimately shows a bi-clonal (ST2 and ST164) population of carbapenem resistant isolates. The study also identifies multiple introductions of GC2 into this hospital setting.

This body of work is comprehensive, highly relevant, and well-written. It leverages up-to-date genomics pipelines to process and analyse an impressive number of genomes, and the analysis is sound. The authors are also commended for having all of the genomics data, including assemblies and reads, available for review in the BioProject.

My only comment relates to the figures. The figures are nicely presented, but are let down by the size of the labelling. Particularly for the phylogenetic trees, the column labels are essentially unreadable because of how small they are, and as a result the reader gets lost in trying to work out what is being shown. Improvements to the size and clarity of the labelling would significantly improve the figures and the reading experience for the reader.

**Thank you for the kind comments. We agree on the clarity of text in figures and have revised the sizes of labels in Fig. 2 and 3. It is difficult to annotate trees containing as many isolates as we present here without reverting to smaller than ideal text, but we hope that the modifications we have made to the revised figures have improved the clarity of the visualization.

We appreciate for your warm work earnestly, and hope that the correction will meet with approval. Once again, thank you very much for your comments and suggestions.

Reviewer #3 (Remarks to the Author):

The manuscript "Longitudinal genomics reveals changes to carbapenem-resistant Acinetobacter baumannii population with the emergence of a globally significant ST164 clone" draws an interesting genomic picture of the globally significant ST164 A. baumannii strains. It is a well-written manuscript and includes much data and analysis. I only have a few general and specific comments as listed below:

- Line 63: The latest update was in April 2024, please use this: https://www.who.int/publications/i/item/9789240093461.

**Thanks for your advice. We have updated this sentence according to your suggestion. Please see “In 2024, the World Health Organisation (https://www.who.int/publications/i/item/9789240093461) reiterated its declaration of

CRAB as a priority organism (Critical group) for which novel therapeutics are urgently required." in line 64-67.

- Line 267: Please include the total number "All (n=?)".

**The total number of "(n=518)" has been added.

- Please replace "pDETAB10::Tn2006" with "pDETAB10/Tn2006" as "::" often indicates insertion/interruption of an element/chromosome/gene/etc.

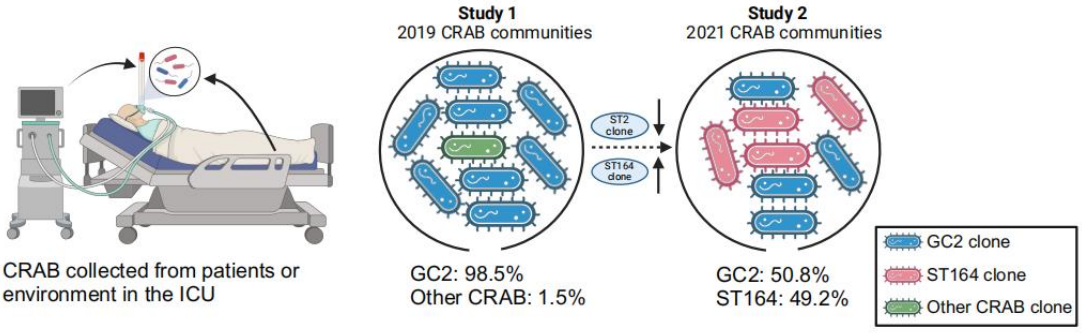
**Has been revised.

- Line 311: Was this the case (Re acquisition of Tn2006/Tn2009 via homologous recombination)?

**We do not have sufficient evidence to declare this here. To determine so would require an extensive examination of the chromosomal segments that flank the transposons, which is virtually impossible given the clonal nature of the population – these chromosomal tracts are identical or near-identical to one another. Detection of homologous recombination events is more easily achieved when they involve more distantly related chromosomes, as was the case in reference 23.

- not sure if the "top part of Panel A in Figure 1" is needed, it is too basic and does not add value. It takes away the attention from the more important parts of this Figure with more meaningful info.

**Thanks for your comment. I agree with your opinion. Now, we have revised the Fig. 1A (please see below) to reflect the true population changes of CRAB with their isolation rate based on the real data in this study. This figure may be able to more vividly show the clonal changes for readers. So, we would like to keep the revised version of Fig. 1A in the manuscript.



- Line 364: It would be interesting to provide more info on the chromosomal location and genetic context of Tn6924 and whether that is possible that this Tn might have been acquired by homologous recombination.

**Tn*6924* is a Tn*7*-like transposon (detailed in the reference cited in-text), and therefore preferentially inserts downstream of the *glmS* gene. This is the case in this ST164 strain, and we have now emphasized this in the manuscript text.

- Line 367: Were Tn2006 and Tn6168 in novel chromosomal positions. This can potentially reveal links to other sequence types with the same transposons. This comment involves Lines 461 and 470-471.

**Thanks for your comment. They were in novel positions, so we cannot infer anything about links to other sequence types from these.

- Line 441: Was KL47 also tracked? Can you please include more info on other STs (from the literature) that include KL47? This would potentially reveal the interaction of their common ancestors.

**We searched "*Acinetobacter baumannii*" and "KL47" and only found one paper (Genomic and Phenotypic Analyses of *Acinetobacter baumannii* Isolates From Three Tertiary Care Hospitals in Thailand) published in 2020[4] describes the STs distribution with different KL types. We downloaded their Table S1 of the properties of *A. baumannii* isolates in their study and the KL47 strains were further screened (Please see below). Results showed majority of the KL47 isolates belonged to GC2 and ST164 clone. In addition, KL47 type was also identified in ST16, ST215 and ST374

isolates (Pasteur MLST scheme). These different ST types, which carry KL47, would potentially reveal the capsular loci interaction of their common ancestors. We have added related content in the discussion section. Please kindly find them in line 552-556.

Sample	Year	Country	ST_Pasteur	ERR	ERS	Contigs	K_Locus
AB15	2016	Thailand	2	ERR2618871	ERS1930165	UFHG01000001-UFHG01000042	KL47
AB51	2016	Thailand	2	ERR2618907	ERS1930201	UFJG01000001-UFJG01000050	KL47
AB78	2016	Thailand	2	ERR2618934	ERS1930228	UFKL01000001-UFKL01000104	KL47
AB81	2016	Thailand	2	ERR2618937	ERS1930231	UFKO01000001-UFKO01000105	KL47
ABAPP-61	2016	Thailand	2	ERR2618957	ERS1930251	UFKT01000001-UFKT01000047	KL47
ABMYSP-195	2016	Thailand	2	ERR2619008	ERS1930302	UFMP01000001-UFMP01000069	KL47
AB1615-09	2016	Thailand	2	ERR2619038	ERS1930332	UFNT01000001-UFNT01000052	KL47
AB2693-09	2016	Thailand	2	ERR2619048	ERS1930342	UFOE01000001-UFOE01000047	KL47
NPRC AB34	2016	Thailand	2	ERR2619086	ERS1930380	UFPU01000001-UFPU01000073	KL47
AB6054	2016	Thailand	16	ERR2619032	ERS1930326	UFNN01000001-UFNN01000113	KL47
AB1931-09	2016	Thailand	16	ERR2619043	ERS1930337	UFNZ01000001-UFNZ01000068	KL47
AB30	2016	Thailand	164	ERR2618886	ERS1930180	UFIQ01000001-UFIQ01000049	KL47
AB87	2016	Thailand	164	ERR2618943	ERS1930237	UFKG01000001-UFKG01000089	KL47
ABJNSP-17	2016	Thailand	164	ERR2618977	ERS1930271	UFLR01000001-UFLR01000075	KL47
ABJNH-92	2016	Thailand	164	ERR2618978	ERS1930272	UFLU01000001-UFLU01000079	KL47
ABAPSP-515	2016	Thailand	164	ERR2618985	ERS1930279	UFMD01000001-UFMD01000063	KL47
ABAPSP-523	2016	Thailand	164	ERR2618987	ERS1930281	UFMH01000001-UFMH01000058	KL47
ABAPP-93	2016	Thailand	164	ERR2618990	ERS1930284	UFMJ01000001-UFMJ01000082	KL47
AB3893	2016	Thailand	164	ERR2619026	ERS1930320	UFNG01000001-UFNG01000108	KL47
AB98	2016	Thailand	215	ERR2618954	ERS1930248	UFKW01000001-UFKW01000296	KL47
AB2069-09	2016	Thailand	374	ERR2619045	ERS1930339	UFON01000001-UFON01000088	KL47

- line 461: What about in publicly available genomes?

**We have compared the Tn*2006* insertion sites with all ST164 publicly available genomes. We found that all Tn*2006* insertions seen in this strain were unique to Hangzhou, as they were not present in the genomes of any ST164 strains isolated elsewhere. However, this section has been removed due to there is no enough genomic evidence to prove.

References

1. Zhao F, *et al.* Description of a Rare Pyomelanin-Producing Carbapenem-Resistant *Acinetobacter baumannii* Strain Coharboring Chromosomal OXA-23 and NDM-1. *Microbiol Spectr* **10**, e0214422 (2022).

2. Richards AM, Abu Kwaik Y, Lamont RJ. Code blue: *Acinetobacter baumannii*, a nosocomial pathogen with a role in the oral cavity. *Mol Oral Microbiol* **30**, 2-15 (2015).

3. Ketter PM, *et al. Acinetobacter baumannii* Gastrointestinal Colonization Is Facilitated by Secretory IgA Which Is Reductively Dissociated by Bacterial Thioredoxin A. *mBio* **9**, (2018).

4. Loraine J, *et al.* Genomic and Phenotypic Analyses of *Acinetobacter baumannii* Isolates From Three Tertiary Care Hospitals in Thailand. *Front Microbiol* **11**, 548 (2020).

At last, we appreciate for the editor' and reviewers' warm work earnestly, and hope that the correction will meet with approval. Once again, thank you very much for your comments and suggestions.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

Remarks:

The authors have addressed the comments and questions raised in my initial review, and their responses have clarified key points in the manuscript. The revisions, both in the content and structure, have significantly improved the overall clarity and presentation of the work. I appreciate your efforts in thoroughly addressing the feedback.

**Thank you for your review. We appreciate your comments and suggestions which are valuable for improving our paper and helpful in promoting the importance of our work.