nature portfolio

Peer Review File

Genomic epidemiology and phylodynamics of Acinetobacter baumannii bloodstream isolates in China

Corresponding Author: Professor Yong-Hong Xiao

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)
Manuscript NCOMMS-24-49644-T analyzes the genomic epidemiology and phylodynamics of Acinetobacter baumannii bloodstream isolates in China over a decade (2011-2021). The study adds novel information to the field and is of general interest but suffers from technical inconsistencies and incorrect analysis of previous specific publications. I am listing below specific queries and compulsory revisions which need to be considered by Authors.
1. It has been demonstrated that Oxford ST92 allelic profile generates because a PCR artifact in the gpi amplicon and that whole genome sequence-based MLST assign these isolates to Oxford ST208 (Hamidian et al. (2017). Problems with the Oxford multilocus sequence typing scheme for Acinetobacter baumannii: do sequence type 92 (ST92) and ST109 exist? J. Clin. Microbiol. 55, 2287–2289. doi: 10.1128/JCM.00533-17). Additional study demonstrated that ST92 has never been found in more than 400 A. baumannii genomes assigned to International (global) clone II (Gaiarsa et al. (2019) Comparative Analysis of the Two Acinetobacter baumannii Multilocus Sequence Typing (MLST) Schemes. Front. Microbiol. 10:930.doi: 10.3389/fmicb.2019.00930). Also, Gaiarsa et al 2019 demonstrated that Oxford ST208, ST191 and ST195 correspond to Pasteur ST2 which have been classified as international clone II (see the Sankey diagram included in Figure 4 of Gaiarsa et al 2019 publication). Because of the above findings, the assignment of A. baumannii genomes belonging to international or global clone II to Oxford CC92 is a mistake which needs to be corrected. Authors should assign ST208, ST191 and ST195 to international clone II and eliminate CC92 and non-CC92 throughout the manuscript.
2. Suppòlementary data set 1: What is the meaning of “STNAN” ?
3. Introduction section, lanes 81-82: “Carbapenem-resistant A. baumannii (CRAB) is considered “critical” among the WHO priority pathogens.” The correct reference to quote instead of refernce nr. 12 is: “WHO Bacterial Priority Pathogens List, 2024: bacterial pathogens of public health importance to guide research, development and strategies to prevent and control antimicrobial resistance. Geneva: World Health Organization; 2024.” ISBN: 978-92-4-009346-1.
4. Introduction section and throughout the manuscript. The following references should be quoted and discussed regarding tolerance to A. baumannii to desiccation and disinfection: a) Lucidi et al, (2024) Pathogenicity and virulence of Acinetobacter baumannii: Factors contributing to the fitness in healthcare settings and the infected host. Virulence. 15:1, 2289769, DOI: 10.1080/21505594.2023.2289769; b) Migliaccio et al. (2022). Inhibition of AdeB, AceI, and AmvA Efflux Pumps Restores Chlorhexidine and Benzalkonium Susceptibility in Acinetobacter baumannii ATCC 19606. Front Microbiol. 12, 790263. doi: 10.3389/fmicb.2021.790263; c) Li et al. (2023). Systematic analyses identify modes of action of ten clinically relevant biocides and antibiotic antagonism in Acinetobacter baumannii. Nat Microbiol. 8(11):1995-2005. doi: 10.1038/s41564-023-01474-z. The term “tolerance to biocides” should be more appropriate than the term “resistance to biocides”.
5. Results section, lines 270-282. Which are the “virulence-related genes” analyzed in ST208, ST191 and ST195 isolates and considered for the analyses reported in Figure 6 ? Because there is no A. baumannii specific virulence genes database available, I am asking if Authors used the virulence factors database (VFDB) at http://www.mgc.ac.cn/VFs/main.htm or built up a novel database. This should be detailed in the Materials and Methods section.
6. In the results section, Authors should analyze the distribution of class B and or class D carbapenemase genes and the genomic sequences responsible for their acquisition such as transposons TN2006, Tn2007 and Tn2009 carrying blaOXA-23 gene
7. Results section line 163: Which is the meaning of “popularity dynamics” ?
8. Supplementary dataset 3. “Isolates that used for experiments”. Do you mean “isolates which have been used in the experiments”?

(Remarks on code availability)
Additional and detailed information on databases and softwares should be provided in the Methods section.

Reviewer #2

(Remarks to the Author)
The authors presented in-depth analyses of 1,506 non-repetitive A. baumannii isolates collected from BSI between 2011 and 2021. 76 sentinel hospitals from 25 provinces were included. The main conclusions were the uneven spatial and temporal distribution of strains. Population analyses showed a shift of STs within CC92 with an increase in ST diversity during the study period, as well as differences in ST distribution between mainland China and globally. The study also focused on the dominant STs of CC92 (ST195, ST208 and ST191) with emphasis on virulence, antibiotic resistance and genome plasticity. The in vivo virulence model showed significantly higher virulence of ST208, and virulence factors (VFs) associated with hypervirulence could explain the observed phenotype. In addition, ST208 showed greater desiccation tolerance compared to ST195 and ST191. This was supported by the finding that the ST208 strain studied has a hydrophilic KL2 capsule, which provides an advantage for survival on abiotic surfaces in the clinical setting. As for the capsules, the authors report that the top three are KL3 , KL2, and KL9 , with the different capsules corresponding to the different STs. When analysing the distribution of KL types, no spatial differences were detected, but temporal changes were described. Genome and phenotype analysis revealed higher antibiotic resistance rates in ST208 than in other analysed STs. The ICEs and transposons were identified as major factors for the acquisition of antibiotic resistance genes.
The evolutionary analyses showed that strain ST208-KL2 is the most recent common ancestor of ST208 strains. The authors conclude that the ability of ST208 to acquire exogenous recombinant genes is greater than that of the other strains and that genomic plasticity is more pronounced, which may be the basis for its adaptation.

The manuscript describes the research with valid methodology that was used for obtaining the results that are adequately discussed. Authors used state of the art methodology and bioinformatics tools that are adequate for this type of research and support presented findings. Figures are well presented and with adequate and detail legends that provide explanations independently from the main text. Supplementary data needed for the thorough and sufficient support for in detailed understanding are provided. In conclusion, methodology is sufficiently detailed and transparent.

Presented conclusions are original, and the results are of interest not only for microbiologists, but also for broader scientific community (e.g. medical scientists, evolutionary biologists, bioinformaticians).

Authors adequately draw out conclusions based on their findings and up to date literature data. Also, they pointed out limitations of the study concerning isolation rate of non-CC92 strains and transmission events of ST208.

Major comment
1. Having in mind that authors have comprehensive collection with strains recovered before, during and after corona-virus pandemic, it would be great for the value of presented study, but also for general knowledge, to see how antibiotic prophylaxis measurements, increased antibiotic treatments and, struggles within clinical settings as well difficulties with epidemiological surveillance during pandemics affected first of all epidemiology and resistome of A. baumannii from BSI. Thus I recommend additional analyses that could derive conclusions on that aspects (e.g. to see eventual shift in epidemiology during pandemic years comparing to previous period, and also to see changes in antibiotic resistance profiles and abundance of ARGs).

Minor comments
1. Please check the statement about total number of STs (140 STs or 149 STs?).
2. Line 132: A total of 140 STs were identified via the Oxford scheme….
3. Line 136: Twenty-six of the 149 identified STs…..
4. Line 163, please revise title (popularity vs. population)
5. Line 279-282, please mitigate the statement
6. Authors should give the reference for MIC breakpoints since for example breakpoint-setting organizations—neither CLSI, FDA, nor EUCAST— provide tigecycline breakpoints for Acinetobacter spp.

(Remarks on code availability)

Reviewer #3

(Remarks to the Author)
This study by Luo et al performs a large analysis of A. baumannii from bloodstream infections in China across several years. The findings are interesting, with some clear differences in epidemiology of major clones between China and other geographic areas. Furthermore the study provides some indications as to why certain A. baumannii lineages may be particularly successful. I believe this provides useful insight to the research community.
There are a few points that I feel should be addressed by the authors.

1. Lines 221-222: The authors say that ST208 and ST195 cluster together whereas ST191 forms a distinct cluster - Figure 3 does not support this. All three STs are reasonably equally separated. Please can this statement be adjusted.

2. Lines 224-225: The authors say that the data in Figure 3 indicate that the other STs in CC92 have derived from the other 3 main STs in CC92. The data do not support this. Firstly the tree is rooted using a single outgroup strain. Use of a different outgroup strain may well alter the shape of the tree. Therefore no inferences should be made about the direction of evolution. Secondly no support values for the tree branches are shown, and therefore no estimation of reliability can be made.

3. Lines 222-223 and Figure 3: It is quite strange that while the ST191, ST195 and ST208 strains are clearly separated from one another, all of the other strains - which are reported to belong to a host of different STs, are all grouped together, and as a group appear to be less genetically diverse than either the ST191 or ST208 groups are. On the face of it, without further explanation, this doesn't seem to make sense. Please can the authors explain this phenomenon.

4. Line 232: The authors have subdivided ST208 into three lineages, with sub-lineage L3 divided further. However, it is not clear what criteria were used for this. For example, looking at Figure 4 there are other sub-clades that appear to be as distinct as those that have been identified as L3 sub-clades, yet they have not been. How were these decisions made? Also please note, on Figure 4 the sub-clades are numbered L3.2.1, L3.2.2 etc. Why are they not called L3.1, L3.2 etc.?

5. Line 330: In the text it says the average number of ARGs in non-CC92 isolates was 19.66, but looking at Figure 7D it looks like its much closer to 30. Please can the authors explain.

6. Lines 373-378 and Figure 8C: The presence of a greater number of SNPs in the ST208 isolates may simply be because ST208 is more distantly related to the reference strain used for SNP calling than the other two STs. Use of a different reference would have produced different results. Therefore describing the number of SNPs in this way and inferring that it is linked with virulence is not robust. Incidentally, the reason for choosing the reference genome is also not given.

7. Lines 551-556: The authors argue that the Oxford MLST scheme is more useful as it discriminates isolates into a greater number of STs. However, they do not mention that a major reason for this is that the Oxford scheme suffers from significant recombination at some of it's loci - something which violates the usual aims of an MLST scheme. This should be included in the discussion to give a balanced view.

8. In general, the figures are not large enough and don't have enough resolution. Some figures are so small/poor resolution that even with zooming in to the electronic version they still can't be read. Figures need to be made larger, with larger colour-code legends and larger labels/axes. Figure 1A and 1C could be supplementary figures and take up a whole page each. In Figure 10, the coloured bar to show what the colour scale means is so thin that the colours can't actually be descerned. Figure S3 - this needs to be rooted - maybe midpoint would be best.

9. In all figures where AMR genes are included, there is no sensible grouping of the genes. For example, all the ADC genes should be together, all the OXA genes should be together etc. Also, for the OXA genes, these need to be grouped by their subtype, so all the different alleles of oxa23 grouped together, all the different alleles of oxaAb (also called oxa51) grouped together etc. The subtype each OXA belongs to can be found on the beta-lactamase database (http://www.bldb.eu/).

(Remarks on code availability)

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Authors correctly addressed queries raised by reviewers and modified the manuscript accordingly.

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)
The authors have adequately addressed all the issues raised and have significantly improved the quality of the manuscript. I therefore recommend the publication of the manuscript.

(Remarks on code availability)

Dear Reviewers,

Thank you very much for your consideration and efforts in reviewing our work. We greatly appreciate your comments and professional advice. Those comments are all valuable and very helpful for revising and improving our paper, as well as the important guiding significance to our researches. We have studied comments carefully and have made correction which we hope meet with approval.

The comments from you are presented in *italics and red*, and we have provided point-by-point responses to each of them. We would like to show the details as follows:

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

Manuscript NCOMMS-24-49644-T analyzes the genomic epidemiology and phylodynamics of Acinetobacter baumannii bloodstream isolates in China over a decade (2011-2021). The study adds novel information to the field and is of general interest but suffers from technical inconsistencies and incorrect analysis of previous specific publications. I am listing below specific queries and compulsory revisions which need to be considered by Authors.

Response: Many thanks for your constructive comments. We have made the revisions based on your comments. Please refer to the responses below, along with the modifications highlighted in the draft.

1. It has been demonstrated that Oxford ST92 allelic profile generates because a PCR artifact in the gpi amplicon and that whole genome sequence-based MLST assign these isolates to Oxford ST208 (Hamidian et al. (2017). Problems with the Oxford multilocus sequence typing scheme for Acinetobacter

baumannii: do sequence type 92 (ST92) and ST109 exist? J. Clin. Microbiol. 55, 2287–2289. doi: 10.1128/JCM.00533-17). Additional study demonstrated that ST92 has never been found in more than 400 A. baumannii genomes assigned to International (global) clone II (Gaiarsa et al. (2019) Comparative Analysis of the Two Acinetobacter baumannii Multilocus Sequence Typing (MLST) Schemes. Front. Microbiol. 10:930.doi: 10.3389/fmicb.2019.00930). Also, Gaiarsa et al 2019 demonstrated that Oxford ST208, ST191 and ST195 correspond to Pasteur ST2 which have been classified as international clone II (see the Sankey diagram included in Figure 4 of Gaiarsa et al 2019 publication). Because of the above findings, the assignment of A. baumannii genomes belonging to international or global clone II to Oxford CC92 is a mistake which needs to be corrected. Authors should assign ST208, ST191 and ST195 to international clone II and eliminate CC92 and non-CC92 throughout the manuscript.

Response: Thank you very much for your professional revision suggestions. We carefully reviewed the research papers you recommended and subsequently removed all references to CC92 and non-CC92 terminology, categorizing Oxford ST191, ST195, and ST208 under IC2.

Current studies employ either the Oxford or the Pasteur typing schemes (PMID 33185494, 35638783, 37655924, 33150386, 36071964, 33461617, 37738153). Each method has its advantages and disadvantages. The Pasteur scheme, which incorporates seven more reliable housekeeping genes, has lower resolution. As a result, most clinically prevalent *A. baumannii* strains are classified as Pasteur ST2. In contrast, the Oxford scheme, although questioned due to its inclusion of the recombination-prone housekeeping gene *gpi*, offers higher resolution and can divide clinical *A. baumannii* strains into multiple ST types. Globally, prevalent clones are usually represented by International Clone (IC) designations, such as IC2, IC1, etc. ICs and clonal complexes (CCs) are identified using the goeBURST algorithm. Both Pasteur ST2 and clinical

prevalent Oxford STs are categorized as IC2, which means the majority of clinically prevalent *A. baumannii* strains belong to IC2.

At the outset of our study, we explored both the Pasteur and Oxford typing schemes (noting each strain's ST types for both schemes in Supplementary Dataset 1). However, we found that the Pasteur scheme assigned the majority of strains to ST2, while the Oxford scheme revealed a greater number of distinct types. To study the evolution and internal variation of prevalent clones, we aimed to strike a balance between the conservativeness and resolution of typing methods. Ultimately, we selected the Oxford scheme for its higher resolution. Further, this scheme also reflects important evolutionary features of *A. baumannii*, making it relevant to our study. The Oxford scheme uncovered diversity within the capsular locus among clonal members, which better enabled us to observe capsule recombination phenomena and their contributions to the evolution of the *A. baumannii* population. This approach aligns well with the central focus of our research.

Our analysis was based on genome sequencing data rather than allele typing by PCR, ensuring accurate identification of the *gpi* gene. In fact, we did not detect the Oxford ST92 type in any genome included in our study (Supplementary Dataset 1). We fully accept your suggestion to remove the reference to CC92. Since ST92 is non-existent, the use of CC92 terminology should indeed be eliminated.

In summary, we completely agree with your suggestions and have made the necessary revisions accordingly. Thank you again for your constructive and professional feedback.

2. Suppòlementary data set 1: What is the meaning of "STNAN" ?

Response: Thank you for highlighting this confusing aspect. STNAN means unidentifiable ST type (next-generation sequencing results revealed incomplete sequences of housekeeping genes used for typing). To avoid ambiguity, we have changed all instances of "STNAN" to "unknown" and have also updated

the other column (Pasteur typing ST) where "NAN" is listed, replacing it uniformly with "unknown."

3. Introduction section, lanes 81-82: "Carbapenem-resistant A. baumannii (CRAB) is considered "critical" among the WHO priority pathogens." The correct reference to quote instead of refernce nr. 12 is: "WHO Bacterial Priority Pathogens List, 2024: bacterial pathogens of public health importance to guide research, development and strategies to prevent and control antimicrobial resistance. Geneva: World Health Organization; 2024." ISBN: 978-92-4-009346-1.

Response: Thank you very much for pointing out this discrepancy. We have now updated ref. 12 with the latest reference you suggested.

4. Introduction section and throughout the manuscript. The following references should be quoted and discussed regarding tolerance to A. baumannii to desiccation and disinfection: a) Lucidi et al, (2024) Pathogenicity and virulence of Acinetobacter baumannii: Factors contributing to the fitness in healthcare settings and the infected host. Virulence. 15:1, 2289769, DOI: 10.1080/21505594.2023.2289769; b) Migliaccio et al. (2022). Inhibition of AdeB, AceI, and AmvA Efflux Pumps Restores Chlorhexidine and Benzalkonium Susceptibility in Acinetobacter baumannii ATCC 19606. Front Microbiol. 12, 790263. doi: 10.3389/fmicb.2021.790263; c) Li et al. (2023). Systematic analyses identify modes of action of ten clinically relevant biocides and antibiotic antagonism in Acinetobacter baumannii. Nat Microbiol. 8(11):1995-2005. doi: 10.1038/s41564-023-01474-z. The term "tolerance to biocides" should be more appropriate than the term "resistance to biocides".

Response: We sincerely appreciate the valuable comments for pointing out the deficiencies in the introduction section and for the suggested references, which have been very helpful to us. We have checked the literature carefully and added the suggested references to the introduction (ref 14-17 in L85). In this

manuscript, "biocide" refers to disinfectants used in hospitals; therefore, we have changed "tolerance to biocide" to "disinfectant tolerance" (L83, L296-314).

5. Results section, lines 270-282. Which are the "virulence-related genes" analyzed in ST208, ST191 and ST195 isolates and considered for the analyses reported in Figure 6 ? Because there is no A. baumannii specific virulence genes database available, I am asking if Authors used the virulence factors database (VFDB) at http://www.mgc.ac.cn/VFs/main.htm or built up a novel database. This should be detailed in the Materials and Methods section.

Response: Thank you for highlighting this question. We used genes from the VFDB database as the "virulence-related genes" in this study and did not construct a new *Acinetobacter baumannii* virulence gene database. To eliminate any concerns, in the Materials and Methods section, we have specified the version of the VFDB database used (L598-600), and in the corresponding part of the Results section, we also clearly stated that the VFDB database was utilized (L261).

6. In the results section, Authors should analyze the distribution of class B and or class D carbapenemase genes and the genomic sequences responsible for their acquisition such as transposons TN2006, Tn2007 and Tn2009 carrying blaOXA-23 gene

Response: Thank you for your suggestion to analyze the distribution of class B and class D carbapenemase genes, as well as the transposons associated with their acquisition. We appreciate the opportunity to clarify our approach in this regard.

Using the beta-lactamase database (http://www.bldb.eu/), we identified several class B and class D carbapenemase genes, including *bla*$_{NDM}$-like, *bla*$_{OXA-23}$-like, *bla*$_{OXA-24}$-like, *bla*$_{OXA-270}$-like, *bla*$_{OXA-51}$-like, and *bla*$_{OXA-58}$-like. We focused specifically on the acquired carbapenemase genes, excluding the

bla$_{OXA-51}$-like genes inherent to *A. baumannii*. In our study, we identified the following carbapenemase genes:

- Class B *bla*$_{NDM}$-like: The *bla*$_{NDM-1}$ gene was found in 6 of the 1,506 isolates. This gene was associated with transposons TnaphA6a (in 3 isolates) and Tn125 (in the other 3 isolates).
- Class D *bla*$_{OXA-23}$-like: We detected two genes within this subfamily, *bla*$_{OXA-23}$ and *bla*$_{OXA-225}$. The *bla*$_{OXA-23}$ gene was present in 1,234 of the 1,506 isolates. Among these, 1,196 isolates carried *bla*$_{OXA-23}$ within a Tn2007 transposon, while 38 isolates carried it within Tn2008. We did not detect Tn2006 or Tn2009, which are known to occur in other regions globally (PMID: 22743015, 28220115, 29091183). The *bla*$_{OXA-225}$ gene was present in 2 isolates, though no mobile genetic elements were identified in its vicinity.
- Class D *bla*$_{OXA-24}$-like: The *bla*$_{OXA-72}$ gene was found in 6 of the 1,506 isolates, and no mobile genetic elements were detected surrounding it.
- Class D *bla*$_{OXA-270}$-like: We identified *bla*$_{OXA-421}$ in 1 isolate without any adjacent mobile genetic elements.
- Class D *bla*$_{OXA-58}$-like: The *bla*$_{OXA-58}$ gene was present in 3 of the 1,506 isolates, also without any mobile genetic elements detected nearby.

These findings highlight the diversity of carbapenemase genes and their associations with various transposons in our sample set. We have incorporated the above results into the manuscript's Results section (L318-326). We appreciate your suggestion and believe this added detail will enhance the reader's understanding of our results.

7. Results section line 163: Which is the meaning of "popularity dynamics" ?

Response: Thank you for highlight this puzzling description. We have changed "popularity dynamics" into "population dynamics" (L160).

8. Supplementary dataset 3. "Isolates that used for experiments". Do you mean "isolates which have been used in the experiments"?

Response: Thank you for your comment. Yes, "Isolates that used for experiments" means "isolates which have been used in the experiments". We have revised in the supplementary dataset 3.

Reviewer #1 (Remarks on code availability):

Additional and detailed information on databases and softwares should be provided in the Methods section.

Response: We feel great thanks for your professional review work on our article. As you are concerned, we carefully checked the databases and software that used in this study, and we added additional and detailed information on databases and software in the Methods section in L588-589, L598-600, L640-642, L647-649, L651-652. The custom code used in this study is freely available at the link https://github.com/ChangMengru/BSIAB_Population_in_China.

Reviewer #2 (Remarks to the Author):

The authors presented in-depth analyses of 1,506 non-repetitive A. baumannii isolates collected from BSI between 2011 and 2021. 76 sentinel hospitals from 25 provinces were included. The main conclusions were the uneven spatial and temporal distribution of strains. Population analyses showed a shift of STs within CC92 with an increase in ST diversity during the study period, as well as differences in ST distribution between mainland China and globally. The study also focused on the dominant STs of CC92 (ST195, ST208 and ST191) with emphasis on virulence, antibiotic resistance and genome plasticity. The in vivo virulence model showed significantly higher virulence of ST208, and virulence factors (VFs) associated with hypervirulence could explain the observed

phenotype. In addition, ST208 showed greater desiccation tolerance compared to ST195 and ST191. This was supported by the finding that the ST208 strain studied has a hydrophilic KL2 capsule, which provides an advantage for survival on abiotic surfaces in the clinical setting. As for the capsules, the authors report that the top three are KL3 , KL2, and KL9 , with the different capsules corresponding to the different STs. When analysing the distribution of KL types, no spatial differences were detected, but temporal changes were described. Genome and phenotype analysis revealed higher antibiotic resistance rates in ST208 than in other analysed STs. The ICEs and transposons were identified as major factors for the acquisition of antibiotic resistance genes.

The evolutionary analyses showed that strain ST208-KL2 is the most recent common ancestor of ST208 strains. The authors conclude that the ability of ST208 to acquire exogenous recombinant genes is greater than that of the other strains and that genomic plasticity is more pronounced, which may be the basis for its adaptation.

The manuscript describes the research with valid methodology that was used for obtaining the results that are adequately discussed. Authors used state of the art methodology and bioinformatics tools that are adequate for this type of research and support presented findings. Figures are well presented and with adequate and detail legends that provide explanations independently from the main text. Supplementary data needed for the thorough and sufficient support for in detailed understanding are provided. In conclusion, methodology is sufficiently detailed and transparent.

Presented conclusions are original, and the results are of interest not only for microbiologists, but also for broader scientific community (e.g. medical scientists, evolutionary biologists, bioinformaticians).

Authors adequately draw out conclusions based on their findings and up to date literature data. Also, they pointed out limitations of the study concerning isolation rate of non-CC92 strains and transmission events of ST208.

Response: We feel great thanks for your professional review work on our article, and we appreciate the reviewer's positive comments, which will facilitate the improvement of our work.

Major comment

1. Having in mind that authors have comprehensive collection with strains recovered before, during and after corona-virus pandemic, it would be great for the value of presented study, but also for general knowledge, to see how antibiotic prophylaxis measurements, increased antibiotic treatments and, struggles within clinical settings as well difficulties with epidemiological surveillance during pandemics affected first of all epidemiology and resistome of A. baumannii from BSI. Thus I recommend additional analyses that could derive conclusions on that aspects (e.g. to see eventual shift in epidemiology during pandemic years comparing to previous period, and also to see changes in antibiotic resistance profiles and abundance of ARGs).

Response: Thank you for your constructive suggestions. In this study, samples were collected via BRICS working group between January 2011 and December 2021. The COVID-19 pandemic in China lasted until the end of 2022, spanning three years. We collected samples from both before and during the first two years of the COVID-19 pandemic but did not include samples from the third year or the post-pandemic period. We analyzed bloodstream infection samples of *Acinetobacter baumannii* from 2020 and 2021 (the first two years of the COVID-19 pandemic in China) and found no significant differences compared to the pre-COVID-19 period in terms of sample quantity, regional distribution, or antibiotic resistance rates (Figure 1, Figure S1, Figure S8 (formerly Figure S7)).

Your question is very insightful, and we share your interest in exploring this further. In future studies, we hope to include strains spanning the pre-, during, and post-COVID-19 periods for a more comprehensive analysis. Thank you once again for your valuable feedback.

Minor comments

1. Please check the statement about total number of STs (140 STs or 149 STs?).

2. Line 132: A total of 140 STs were identified via the Oxford scheme….

3. Line 136: Twenty-six of the 149 identified STs…..

Response to *1, 2 and 3*: We sincerely thank the reviewer for careful reading. We were really sorry for our careless mistakes. We identified 149 STs in this study. We have checked the data and revised 140 STs into 149 STs in L129.

4. Line 163, please revise title (popularity vs. population)

Response: Thank you for pointing out this error. We have revised "popularity" into "population" (L160).

5. Line 279-282, please mitigate the statement

Response: Thank you for your suggestion. We have revised these sentences to be more concise (L274-276).

6. Authors should give the reference for MIC breakpoints since for example breakpoint-setting organizations—neither CLSI, FDA, nor EUCAST— provide tigecycline breakpoints for Acinetobacter spp.

Response: Thank you for your comments. We have included the "Antimicrobial susceptibility testing" part in the Methods section (L682-691). The results were interpreted according to the Clinical and Laboratory Standards Institute and European Committee (CLSI, 2023) or Antimicrobial Susceptibility Testing v.13.0 (http://www.eucast.org/clinical_breakpoints/). Carbapenem resistance was defined as a minimum inhibitory concentration (MIC) of ≥8 mg/L for imipenem or meropenem. Tigecycline resistance was defined as a MIC of ≥8 mg/L. Polymyxin resistance was defined as a MIC of >2 mg/L.

Reviewer #3 (Remarks to the Author):

This study by Luo et al performs a large analysis of A. baumannii from bloodstream infections in China across several years. The findings are interesting, with some clear differences in epidemiology of major clones between China and other geographic areas. Furthermore the study provides some indications as to why certain A. baumannii lineages may be particularly successful. I believe this provides useful insight to the research community. There are a few points that I feel should be addressed by the authors.

Response: We really appreciate the reviewer's positive comments, which will facilitate the improvement of our work.

1. Lines 221-222: The authors say that ST208 and ST195 cluster together whereas ST191 forms a distinct cluster - Figure 3 does not support this. All three STs are reasonably equally separated. Please can this statement be adjusted.

Response: Thank you for pointing out this, we fully agree with your suggestion. We deleted the inappropriate statement "ST208 and ST195 cluster together, whereas ST191 forms a distinct cluster."

Further, we want to clarify that the phylogenetic tree presented in Figure 3 uses the *A. baumannii* XH857 strain (accession number: NZ_CP014540.1, Oxford ST806) as the outgroup. we performed 100 rapid bootstrap replications when constructing the phylogenetic tree, and we have now included the bootstrap values in the revised Figure 3. The support values for the main branches range from 82% to 100%, indicating a high level of confidence in these branches.

2. Lines 224-225: The authors say that the data in Figure 3 indicate that the other STs in CC92 have derived from the other 3 main STs in CC92. The data do not support this. Firstly the tree is rooted using a single outgroup strain. Use

of a different outgroup strain may well alter the shape of the tree. Therefore no inferences should be made about the direction of evolution. Secondly no support values for the tree branches are shown, and therefore no estimation of reliability can be made.

Response: Thank you. We greatly appreciate your insightful comments.

The phylogenetic tree presented in Figure 3 of our study uses the *A. baumannii* XH857 strain (accession number: NZ_CP014540.1, Oxford ST806) as the outgroup. However, during the tree construction process, we also tested using three IC1 strains (AB0057, AYE, AB5075), as well as strains from ST191, ST195, ST208, and other ST types. The branching positions of these strains on the tree were consistent with those obtained using *A. baumannii* XH857 (accession number: NZ_CP014540.1, Oxford ST806). Given that IC1 strains are phylogenetically distant from IC2 strains, they did not effectively display branch lengths within the tree. In contrast, *A. baumannii* XH857, being a non-IC2 strain with closer relatedness to IC2 strains, allowed for a clearer representation of the internal structure and branch lengths of the IC2 phylogenetic tree. Thus, we selected *A. baumannii* XH857 as the outgroup for our study.

Moreover, we noted that other studies on IC2 (GC2) have also chosen *A. baumannii* XH857 as the outgroup for phylogenetic tree construction. Please refer to Fig. 3 in the following study for reference: https://academic.oup.com/mbe/article/37/2/563/5601619. Therefore, we believe that the outgroup chosen in our study is reliable.

In addition, we performed 100 rapid bootstrap replications when constructing the phylogenetic tree, and we have now included the bootstrap values in the revised Figure 3. The support values for the main branches range from 82% to 100%, indicating a high level of confidence in these branches.

Regarding your comment on inferring evolutionary origins, we fully agree with your suggestion. Indeed, the origins cannot be directly inferred from the

clustering pattern of the tree. We have revised the relevant statement in line L213-221to reflect this.

3. Lines 222-223 and Figure 3: It is quite strange that while the ST191, ST195 and ST208 strains are clearly separated from one another, all of the other strains - which are reported to belong to a host of different STs, are all grouped together, and as a group appear to be less genetically diverse than either the ST191 or ST208 groups are. On the face of it, without further explanation, this doesn't seem to make sense. Please can the authors explain this phenomenon.

Response: Thank you for the valuable comments. Regarding the maximum likelihood tree presented in Figure 3, we addressed the reliability of the tree-building method as well as the biological significance of the phylogenetic tree structure below. Furthermore, we provided a more detailed visualization to depict the internal structure of the branch for "other ST types", which shows greater genetic diversity compared to the branches of ST191/ST195/ST208 (the following Figure).

Tree-building method: The maximum likelihood tree in Figure 3 was constructed using a non-redundant set of 1,040 IC2 (formerly referred to as CC92) strains, rooted with an outgroup method. For the choice of the outgroup, we tested three IC1 strains (AB0057, AYE, AB5075), and found that the positions of ST191, ST195, ST208, and other ST branches on the phylogenetic tree remained consistent with our study's choice of *A. baumannii* XH857 (accession number: NZ_CP014540.1, Oxford ST806). Because IC1 strains are distantly related to IC2, they do not effectively showcase branch lengths in the phylogenetic tree. *A. baumannii* XH857, however, is a non-IC2 strain more closely related to IC2 strains, allowing us to clearly display both internal structure and branch lengths within IC2. Consequently, we used *A. baumannii* XH857 as the outgroup in this study. Additionally, we observed that other studies on IC2 (GC2) have used *A. baumannii* XH857 as an outgroup, as seen in Figure 3 of this paper: Hu, D., Liu, B., Wang, L., & Reeves, P. R. (2020).

Living trees: high-quality reproducible and reusable construction of bacterial phylogenetic trees. Molecular biology and evolution, 37(2), 563-575. Thus, the outgroup selection in our study is well-supported.

Distinct branches for ST191, ST195, and ST208: In the maximum likelihood tree in Figure 3, ST191, ST195, and ST208 show clear differentiation and occupy distinct branches, while strains of other ST types within IC2 cluster into a single branch. Coupled with other analytical results in this study, this suggests that ST191, ST195, and ST208 represent clonally expanding, successful strains in China, likely due to adaptive advantages. These three STs have persisted over time in China and, under selective pressures in hospital environments (such as antibiotics), have accumulated numerous mutations. This accumulation of mutations may drive their clear divergence in the evolutionary process. In contrast, non-dominant STs lack the specific selective pressures faced by these dominant strains and have not accumulated sufficient mutations to promote clonal expansion, resulting in genetic similarity and lower genetic diversity. Consequently, these strains form a distinct, tightly clustered branch in the phylogenetic tree. Additionally, factors such as evolutionary origin and evolutionary pathways may influence the genetic differences between dominant and non-dominant STs. This phenomenon likely reflects the combined effects of multiple factors.

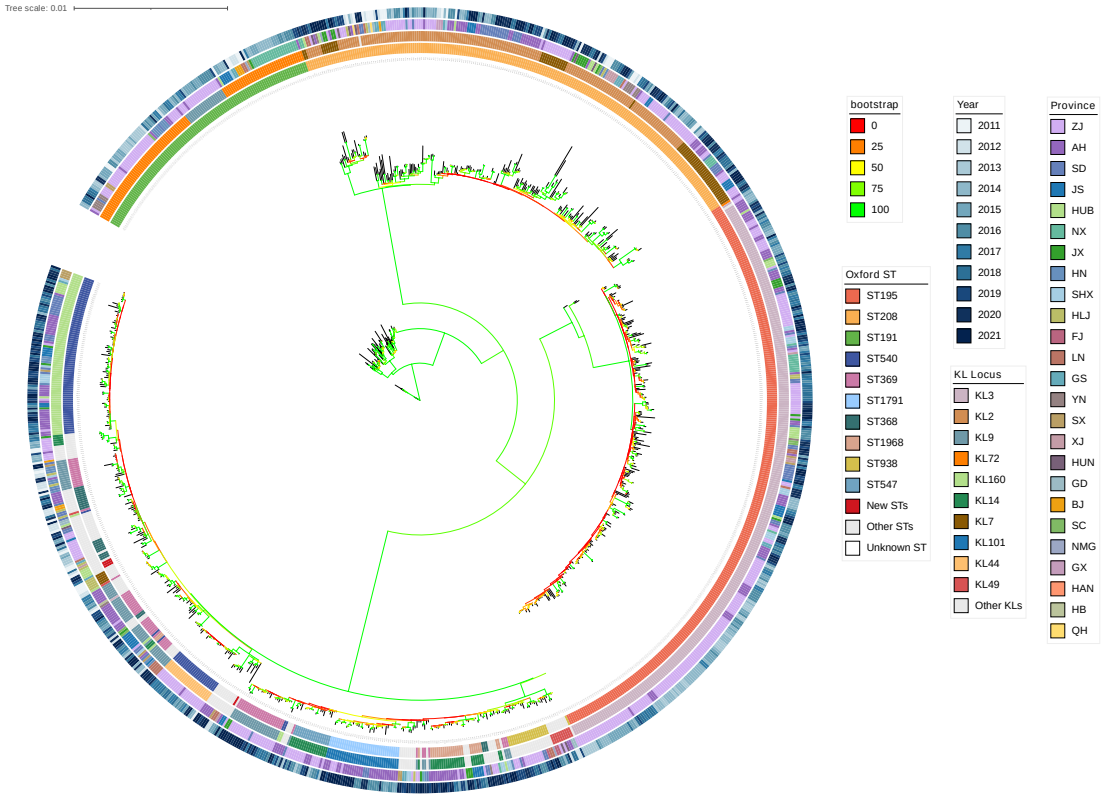

Figure. Maximum likelihood phylogeny of IC2 BSI-causing *A. baumannii* isolates in this study. The phylogenetic tree is rooted according to the *A. baumannii* XH857 (accession number: NZ_CP014540.1) outgroup. Branch lengths represent the frequency of nucleotide substitutions per site, as indicated by the scale bar. (This is the circle type of Figure 3 in the manuscript.)

4. Line 232: The authors have subdivided ST208 into three lineages, with sub-lineage L3 divided further. However, it is not clear what criteria were used for this. For example, looking at Figure 4 there are other sub-clades that appear to be as distinct as those that have been identified as L3 sub-clades, yet they have not been. How were these decisions made? Also please note, on Figure 4 the sub-clades are numbered L3.2.1, L3.2.2 etc. Why are they not called L3.1, L3.2 etc.?

Response: Thank you for your valuable comments. In this study, the detailed subdivision of the ST208 lineage was conducted using a phylogenetic inference

of lineages through the hierarchical Bayesian population structure analysis (hierBAPS) model in R (rHierBAPs), with the parameters set as follows: maximum depth of 5 and initial population size of 50. Specifically, we performed hierarchical Bayesian population structure inference on the non-redundant IC2 phylogenetic tree (Fig. 3). We then collapsed the branches corresponding to ST191, ST195, and other ST types, and focused on enlarging the internal lineage structure of the ST208 branch. This analysis revealed that ST208 could be divided into three lineages (L1-L3), with L3 further subdivided into 11 sub-lineages.

Regarding the ambiguous naming of sub-clades in Figure 4, we have revised the figure and clearly labeled the additional sub-lineages within L3. Additionally, the following corrections have been made as per your suggestions: Line 232: "three sub-lineages" has been corrected to "11 sub-lineages." Line 236: "L3.2 (n=27)" has been updated to "L3.1 (n=13), L3.8 (n=6), L3.10 (n=4), L3.11 (n=4)." Thank you once again for your insightful suggestions.

5. Line 330: In the text it says the average number of ARGs in non-CC92 isolates was 19.66, but looking at Figure 7D it looks like its much closer to 30. Please can the authors explain.

Response: Thank you very much for your thorough review and insightful comments. We have revised the figure according to your comment. In Figure 7, the average ARG count for non-IC2 (formerly non-CC92) isolates is mentioned as 19.59, which is reflected in the text on L318. Figure 7B, however, displays the ARG counts for the prevalent IC2 clones ST191, ST195, and ST208, which indeed average closer to 30. We have now also adjusted and corrected the results based on the typing suggestions from Reviewer #1. The results show that the number of ARGs in IC2 is 30.81±3.06, while in non-IC2 it is 19.59±3.32, which is consistent with what is presented in Figure 7D. We appreciate your attention to detail, and hope this clarification addresses your concern.

6. Lines 373-378 and Figure 8C: The presence of a greater number of SNPs in the ST208 isolates may simply be because ST208 is more distantly related to the reference strain used for SNP calling than the other two STs. Use of a different reference would have produced different results. Therefore describing the number of SNPs in this way and inferring that it is linked with virulence is not robust. Incidentally, the reason for choosing the reference genome is also not given.

Response: Thank you for your insightful feedback. We appreciate your point regarding the choice of reference genome and the potential influence it has on SNP analysis results. To clarify, our intention was not to link the number of SNPs directly with virulence but rather to use SNP analysis to demonstrate the differences in the genetic diversity of prevalent ST types.

The reference genome used in this study is *A. baumannii* BJAB07104 (accession number: NC_021726.1), a bloodstream-derived IC2 strain isolated in 2007 from the PLA General Hospital in Beijing, China (https://doi.org/10.1371/journal.pone.0066584), and its Oxford sequence type is ST368. The reasons for selecting *A. baumannii* BJAB07104 as the reference genome are as follows:

- This strain has been included in the RefSeq database and has a complete, high-quality genome sequence.
- All the *A. baumannii* strains used in this study are bloodstream isolates from clinical settings in China, collected over a span of 2011-2021. Since this strain was isolated earlier than the strains in our study and shares a similar clinical background, it provides essential historical benchmark information for our analysis of genetic evolution and epidemiology.
- *A. baumannii* BJAB07104 belongs to the IC2 lineage, and 1,231 out of 1,506 strains in this study are IC2. Its genome structure and genetic distance are closely related to 81.7% of the strains in our study, which facilitates the genetic evolutionary analysis.

- Previous research on adaptive evolution (https://doi.org/10.1093/pnasnexus/pgad079) and genomic studies of *A. baumannii* (https://www.nature.com/articles/srep26930) have also used *A. baumannii* BJAB07104 as a reference genome.

Considering all these aspects, *A. baumannii* BJAB07104 was chosen as the reference genome for this study. However, this choice is not fixed. As seen in various research methodologies on *A. baumannii*, there is no perfect reference genome that fits all analytical approaches. Researchers need to select an appropriate reference genome based on their specific study goals and genome data.

Additionally, we have considered the evolutionary distances between the reference genome *A. baumannii* BJAB07104 and ST208, ST191, and ST195. *A. baumannii* BJAB07104 belongs to ST368, and based on the IC2 phylogenetic tree, it is closest in evolutionary distance to ST195 and furthest from ST191. Therefore, the choice of reference genome is not the main factor that affects the number of SNP in ST208 higher than that in ST191 and ST195.

7. Lines 551-556: The authors argue that the Oxford MLST scheme is more useful as it discriminates isolates into a greater number of STs. However, they do not mention that a major reason for this is that the Oxford scheme suffers from significant recombination at some of it's loci - something which violates the usual aims of an MLST scheme. This should be included in the discussion to give a balanced view.

Response: Thank you very much for your insightful feedback.

Current studies employ either the Oxford or Pasteur typing schemes (PMID 33185494, 35638783, 37655924, 33150386, 36071964, 33461617, 37738153). Each method has its advantages and disadvantages. The Pasteur scheme, which incorporates seven more reliable housekeeping genes, has lower resolution. As a result, most clinically prevalent *A. baumannii* strains are classified as Pasteur ST2. In contrast, the Oxford scheme, although questioned

due to its inclusion of the recombination-prone housekeeping gene *gpi*, offers higher resolution and can divide clinical *A. baumannii* strains into multiple ST types. Globally, prevalent clones are usually represented by International Clone (IC) designations, such as IC2, IC1, etc. ICs and clonal complexes (CCs) are identified using the goeBURST algorithm. Both Pasteur ST2 and clinical prevalent Oxford STs are categorized as IC2, which means the majority of clinically prevalent *A. baumannii* strains belong to IC2.

At the outset of our study, we carefully considered both the Pasteur and Oxford MLST schemes, and we recorded the ST types for each strain under both systems in Supplementary Dataset 1. While we observed that the Pasteur scheme predominantly classified the strains as ST2, the Oxford scheme provided a higher resolution by revealing greater diversity among sequence types. Since our primary objective was to track the evolution and shifts within prevalent clones, we ultimately decided to use the Oxford scheme due to its finer discrimination. Additionally, the Oxford scheme allowed us to observe greater diversity within the capsular locus of clonal members, which proved crucial for investigating capsule recombination events and their impact on the evolution of *A. baumannii* populations. This focus on capsular diversity aligns closely with the central aims of our study.

We appreciate your reference to the recombination issues present in some of the loci (such as *gpi* gene) within the Oxford scheme, which indeed may conflict with the traditional objectives of an MLST scheme. However, we believe that when studying the genomic epidemiology and evolutionary trends of pathogenic bacteria, it is essential to strike a balance between the conservativeness and resolution of typing methods to better serve the research objectives. In fact, some recent studies have moved away from traditional MLST typing in favor of genome-wide or core-genome approaches such as cgMLST (core-genome MLST) (PMID: 34220740, 36989679). While it is true that the *gpi* region undergoes recombination, this recombination reflects

important evolutionary features of *A. baumannii.* After careful consideration, we chose the Oxford scheme because it best aligns with the goals of our study.

In light of this, we have revised the Discussion section to provide a more balanced perspective and included a note about this issue (L538-547). In summary, we fully agree with your suggestions and have made the necessary revisions. We are grateful for your constructive and professional feedback, which has been invaluable to improving our manuscript.

8. In general, the figures are not large enough and don't have enough resolution. Some figures are so small/poor resolution that even with zooming in to the electronic version they still can't be read. Figures need to be made larger, with larger colour-code legends and larger labels/axes. Figure 1A and 1C could be supplementary figures and take up a whole page each. In Figure 10, the coloured bar to show what the colour scale means is so thin that the colours can't actually be descerned. Figure S3 - this needs to be rooted - maybe midpoint would be best.

Response: Thank you for your valuable feedback regarding the figures in our manuscript. We have thoroughly reviewed all the figures in light of your suggestions and have made several improvements to enhance their resolution and readability.

We greatly appreciate your suggestion to improve the visual clarity of Figures 1A and 1C. However, after careful consideration, we believe that Figure 1A is crucial for supporting the results presented in the manuscript. Therefore, we feel it is more appropriate to keep Figures 1A within the main text. We have adjusted the size of the figure to enhance their visual quality as much as possible. As suggested by you, we have changed Figure 1C to supplementary Figure S2. In Figure 10, we have enlarged the color bar to ensure that the colors representing the number of mobile genetic elements (MGEs) detected per genome are more distinguishable, as indicated by the accompanying legend. We have adjusted Figure S3 (now Figure S4) by using a midpoint rooting

method, as you recommended, to enhance its clarity.

In addition, we also have made the following adjustments:

- Figure 1DE, Figure 2, Figure S4 (formerly Figure S3), Figure S5B (formerly Figure S4B), and Figure S8B (formerly Figure S7B) have been redrawn according to the IC2 classification.
- Bootstrap values have been added to Figure 3 and Figure S7 (formerly Figure S6), and the tree branches are color-coded accordingly.
- Figure 4 has been relabeled based on the sub-lineages.
- Figure 7D and Figure S8A (formerly Figure S7A) have been recalculated and the classification of the *bla* genes has been marked.
- For Figure 10ABCD, the legend has been bolded, and we have improved the overall visual clarity of the figures as much as possible.
- The previously unmarked section D in Figure 11 has now been labeled.
- The year labels on the right side of Figure S1E have been corrected to display fully.
- Figure S9 (formerly Figure S8) has been labeled with A, B, C, and D.

We have carefully revised the sizes and clarity of all figures in the manuscript to ensure they are of high quality and resolution. We appreciate your constructive input, which has helped us improve the quality of our figures. Thank you for your thoughtful review.

9. In all figures where AMR genes are included, there is no sensible grouping of the genes. For example, all the ADC genes should be together, all the OXA genes should be together etc. Also, for the OXA genes, these need to be grouped by their subtype, so all the different alleles of oxa23 grouped together, all the different alleles of oxaAb (also called oxa51) grouped together etc. The subtype each OXA belongs to can be found on the beta-lactamase database (http://www.bldb.eu/).

Response: Thank you for your valuable comments. We have made adjustments to the AMR gene grouping in Figures 7D and S8A (formerly Figure

S7A), organizing the AMR genes more systematically. All ADC genes have been grouped together, all *bla*$_{OXA}$ genes have been placed in a single group, and the *ade* family genes have been grouped accordingly. Additionally, we consulted the beta-lactamase database (http://www.bldb.eu/) to identify carbapenemase genes, including *bla*$_{NDM}$-like, *bla*$_{OXA-23}$-like, *bla*$_{OXA-24}$-like, *bla*$_{OXA-270}$-like, *bla*$_{OXA-51}$-like, and *bla*$_{OXA-58}$-like. These classifications have been annotated and marked in both Figures 7D and Figure S8A (formerly Figure S7A).

Thanks again for your efforts in reviewing this manuscript and for the valuable suggestions in revision. Thank you very much for your attention and time. Look forward to hearing from you.

Yours sincerely,

Yonghong Xiao

25. Oct. 2024/10/23

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