nature portfolio

Peer Review File

Increasing polymyxin resistance in clinical carbapenem-resistant Klebsiella pneumoniae strains in China between 2000 and 2023

Corresponding Author: Professor Rong Zhang

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)
Dear Author
Thank you for your manuscript submission. The manuscript is interesting; however, a revision is needed as below:

1. Please do read and add the following papers to References section to have fruitful Introduction and Discussion sections:

Emerging multidrug-resistant bacterial pathogens "superbugs": A rising public health threat. Front Microbiol. 2023 Feb 1;14:1135614. doi: 10.3389/fmicb.2023.1135614. PMID: 36819057; PMCID: PMC9930894.

Carbapenem-Resistant Klebsiella pneumoniae: Virulence Factors, Molecular Epidemiology and Latest Updates in Treatment Options. Antibiotics (Basel). 2023 Jan 21;12(2):234. doi: 10.3390/antibiotics12020234. PMID: 36830145; PMCID: PMC9952820.

Pan-Genome Plasticity and Virulence Factors: A Natural Treasure Trove for Acinetobacter baumannii. Antibiotics (Basel). 2024 Mar 14;13(3):257. doi: 10.3390/antibiotics13030257. PMID: 38534692; PMCID: PMC10967457.

Molecular Detection of gyrA Mutation in Clinical Strains of Klebsiella pneumoniae. Iran J Public Health. 2022 Oct;51(10):2334-2339. doi: 10.18502/ijph.v51i10.10992. PMID: 36415795; PMCID: PMC9647615.

Virulence factors, antibiotic resistance patterns, and molecular types of clinical isolates of Klebsiella Pneumoniae. Expert Rev Anti Infect Ther. 2022 Mar;20(3):463-472. doi: 10.1080/14787210.2022.1990040. Epub 2021 Oct 28. PMID: 34612762.

Metallo-ß-lactamases: a review. Mol Biol Rep. 2020 Aug;47(8):6281-6294. doi: 10.1007/s11033-020-05651-9. Epub 2020 Jul 11. PMID: 32654052.

Antimicrobial Agents and Urinary Tract Infections. Curr Pharm Des. 2019;25(12):1409-1423. doi: 10.2174/1381612825999190619130216. PMID: 31218955.

Prevalence of tetracycline resistance genes tet (A, B, C, 39) in Klebsiella pneumoniae isolated from Tehran, Iran. Iranian Journal of Medical Microbiology. 2022 Apr 10;16(2):141-7.

2. Please do revise the Keywords as below:
Polymyxin resistance; carbapenem resistance; Klebsiella pneumoniae; clinical isolates;
molecular epidemiology; phylogenetic analysis

3. Please do add the type of clinical samples: urine, blood, etc.

4. Please do write the antibiotic sensitive test section in accordance with standard format: including the name of antibiotics (standard unit/ name of Company/Country)

5. Please do add a flow chart to show all the procedures done within the current study.

6. It would be very interesting if the age range and the gender of the patients were included, too. I recommend to add these items to your study, too.

Reviewer #2

(Remarks to the Author)

Thank you to Xie et al., for submitting the work entitled: “Polymyxin resistance in clinical carbapenem-resistant Klebsiella pneumoniae strains in China: molecular epidemiological study from 2000~2023”

Here, the authors investigated the prevalence of polymyxin resistance in carbapenem-resistant Klebsiella pneumoniae (CRKP) strains in China and the underlying molecular mechanisms leading to resistance. Of the 4455 clinical CRKP strains collected, 112 were found to be polymyxin resistant, which underwent further molecular and phenotypic analysis. Following sequencing, draft assemblies of these strains were analysed with Kleborate to determine AMR and virulence gene content, and phylogenetic relationships were determined with Snippy. Six strains were further phenotypically characterised for hypermucoviscosity, capsule production and virulence in mice.

This is an impressive collection with accompanying sequence data and is a welcome resource to the community. However, quality of the data analysis is lacking, and some results are difficult to interpret if not completely omitted, therefore I suggest major revisions. Detailed comments are as follows:

Major comments
There needs to be a supplementary table listing every strain, its relative NCBI accession, assembly statistics and other genotypic data (Kleborate, Kaptive, etc). This genotypic data is only summarised in text and must be provided.

There are no methods sections on the mucoviscosity assay (Fig 4b), capsule production (Fig 4c) or mouse sepsis model (Fig 4d); these must be included in detail for reproducibility.

Why were only 6 strains chosen for the phenotypic analysis? What was the rationale behind choosing these 6 strains? Do they truly reflect the diversity of the dataset of the polymyxin resistant CRKP in this study?

The authors found that the cause of polymyxin resistance in these CRKP isolates was mutation-mediated and not via horizontal transfer of MCR-1; could the authors add some further clarity/thoughts about:
a) Why these mutations first appeared in 2014 despite collection since 2003; were there any changes to clinical procedure that may have caused this.
b) Why did mutation rates increase over time, yet the proportion of strain backgrounds remain the same (except 2014 and 2017)?

Line 322: The strain 14-109 was used as a reference for core-SNV alignment and comparison with Snippy. This assembly (ASM3967506v1, GCA_039675065.1) is fragmented across 108 contigs and is therefore SNV information may be lost in genomic regions that frequently assemble poorly, such as the K-locus. What was the rationale behind using this strain as a reference when most strains are ST11? There are completed ST11 genomes available which would make a better reference.

Line 323: Snippy produces a core-SNP alignment, but not a phylogenetic tree. What algorithm was used to produce the phylogenetic tree? Please list the software, version, algorithm and method used to root the tree.

Minor comments
Line 30: “Totally 112 polymyxin-resistant CRKP isolates were identified.” Replace “Totally” with “In total,”

Line 296: How were the polymyxin-resistant CRKP isolates identified initially, this is not made clear.

Line 319: K- and O-locus calls are reported so Kaptive needs to be cited (Wyres et al., MGen 2016) as per the Kleborate documentation (https://kleboratemodular.readthedocs.io/en/latest/#citations).

Line 324: How were complete plasmid sequences determined from short-read draft assemblies?

Lines 249-251: There is conflicting evidence on whether polymyxin resistance results in increased enhanced virulence in Kp; the study cited here used a different lineage (ST258) which is not seen in this geography and the G. mellonella model is not able to distinguish between hv and classical Kp (Russo and MacDonald, mSphere, 2020). In addition, another study has shown that other polymyxin mutations (PhoQ) can sensitize Kp to IgM-mediated complement killing (van der Lans, S.P.A., et al., Sci Rep 2023).

Table 2: Why were the strains not tested against colistin (Polymyxin E)?

Fig1: The resolution of this figure is too low making it difficult to read. Please increase the resolution of this figure.

Fig4a: The legend is recycled so multiple strains have the same key. This is bad practice for figures and make it impossible to interpret the data. One solution would be to cluster the plasmid contigs so only non-redundant sequences are compared, reducing the number of rings.

Fig4bcd: The legend is on the far right, so it is unclear that it refers to b and c too. The legend should be in solid colours and put above all figures, so it is clear the key refers to all panels.

It would be good to see a temporal breakdown of the clones over the sampling periods to demonstrate the proportion of clones over time. This can be represented as a ridge plot, with time on the X-axis and clone proportion on the Y-axis.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Dear Author
Thank you for revisions.

Reviewer #2

(Remarks to the Author)
Thanks to the authors for addressing my previous comments. This version is an improvement and I have no further comments.

Response to Review Comments

Reviewers' comments:

Reviewer #1 (Remarks to the Author):
Dear Author
Thank you for your manuscript submission. The manuscript is interesting; however, a revision is needed as below:

1. Please do read and add the following papers to References section to have fruitful Introduction and Discussion sections:

Emerging multidrug-resistant bacterial pathogens "superbugs": A rising public health threat. Front Microbiol. 2023 Feb 1;14:1135614. doi: 10.3389/fmicb.2023.1135614. PMID: 36819057; PMCID: PMC9930894.

Carbapenem-Resistant Klebsiella pneumoniae: Virulence Factors, Molecular Epidemiology and Latest Updates in Treatment Options. Antibiotics (Basel). 2023 Jan 21;12(2):234. doi: 10.3390/antibiotics12020234. PMID: 36830145; PMCID: PMC9952820.

Pan-Genome Plasticity and Virulence Factors: A Natural Treasure Trove for Acinetobacter baumannii. Antibiotics (Basel). 2024 Mar 14;13(3):257. doi: 10.3390/antibiotics13030257. PMID: 38534692; PMCID: PMC10967457.

Molecular Detection of gyrA Mutation in Clinical Strains of Klebsiella pneumoniae. Iran J Public Health. 2022 Oct;51(10):2334-2339. doi: 10.18502/ijph.v51i10.10992. PMID: 36415795; PMCID: PMC9647615.

Virulence factors, antibiotic resistance patterns, and molecular types of clinical isolates of Klebsiella Pneumoniae. Expert Rev Anti Infect Ther. 2022 Mar;20(3):463-472. doi: 10.1080/14787210.2022.1990040. Epub 2021 Oct 28. PMID: 34612762.

Metallo-ß-lactamases: a review. Mol Biol Rep. 2020 Aug;47(8):6281-6294. doi: 10.1007/s11033-020-05651-9. Epub 2020 Jul 11. PMID: 32654052.

Antimicrobial Agents and Urinary Tract Infections. Curr Pharm Des. 2019;25(12):1409-1423. doi: 10.2174/1381612825999190619130216. PMID: 31218955.

Prevalence of tetracycline resistance genes tet (A, B, C, 39) in Klebsiella pneumoniae isolated from Tehran, Iran. Iranian Journal of Medical Microbiology. 2022 Apr 10;16(2):141-7.

Response: We have read the above papers. Four papers have been cited in the Discussion and added in the Reference section.

2. Please do revise the Keywords as below:
Polymyxin resistance; carbapenem resistance; Klebsiella pneumoniae; clinical isolates; molecular epidemiology; phylogenetic analysis

Response: Revised accordingly.

3. Please do add the type of clinical samples: urine, blood, etc.

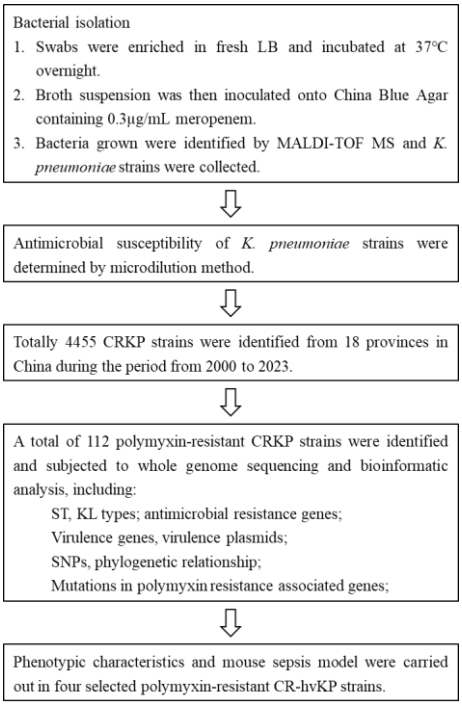
Response: Sorry, we cannot provide the type of clinical samples since many of them are missing.

4. Please do write the antibiotic sensitive test section in accordance with standard format: including the name of antibiotics (standard unit/ name of Company/Country)

Response: The name of antibiotics has been added. The sentence has been revised to "The susceptibility of polymyxin-resistant CRKP isolates to 15 commonly used antibiotics (including amikacin, aztreonam, cefepime, cefmetazole, cefoperazone-sulbactam, cefotaxime, ceftazidime, ceftazidime-avibactam, ciprofloxacin, ertapenem, meropenem, piperacillin-tazobactam, polymyxin B and tigecycline (Sigma-Aldrich, USA)) was determined using broth microdilution method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines."

5. Please do add a flow chart to show all the procedures done within the current study.

Response: A flow chart showing the procedures has been added in Supplementary Materials as follows.



Supplementary Fig S1. Flow chart diagram showing the isolation and analysis of polymyxin-resistant CRKP strains in this study.

6. It would be very interesting if the age range and the gender of the patients were included, too. I recommend to add these items to your study, too.

Response: Sorry, we cannot provide the age and gender of the patients, because according to the hospital's relevant regulations, patients' clinical information cannot be disclosed.

Reviewer #2 (Remarks to the Author):

Thank you to Xie et al., for submitting the work entitled: “Polymyxin resistance in clinical carbapenem-resistant Klebsiella pneumoniae strains in China: molecular epidemiological study from 2000~2023”

Here, the authors investigated the prevalence of polymyxin resistance in carbapenem-resistant Klebsiella pneumoniae (CRKP) strains in China and the underlying molecular mechanisms leading to resistance. Of the 4455 clinical CRKP strains collected, 112 were found to be polymyxin resistant, which underwent further molecular and phenotypic analysis. Following sequencing, draft assemblies of these strains were analysed with Kleborate to determine AMR and virulence gene content, and phylogenetic relationships were determined with Snippy. Six strains were further phenotypically characterised for hypermucoviscosity, capsule production and virulence in mice.

This is an impressive collection with accompanying sequence data and is a welcome resource to the community. However, quality of the data analysis is lacking, and some results are difficult to interpret if not completely omitted, therefore I suggest major revisions. Detailed comments are as follows:

Major comments:
1. There needs to be a supplementary table listing every strain, its relative NCBI accession, assembly statistics and other genotypic data (Kleborate, Kaptive, etc). This genotypic data is only summarised in text and must be provided.

Response: A supplementary table listing every strain, its NCBI accession, assembly statistics, and genotypic data is provided.

2. There are no methods sections on the mucoviscosity assay (Fig 4b), capsule production (Fig 4c) or mouse sepsis model (Fig 4d); these must be included in detail for reproducibility.

Response: The methods of mucoviscosity assay, capsule production and mouse sepsis model have been added to the Methods section.

3. Why were only 6 strains chosen for the phenotypic analysis? What was the rationale behind choosing these 6 strains? Do they truly reflect the diversity of the dataset of the polymyxin resistant CRKP in this study?

Response: Totally 46 polymyxin-resistant CRKP isolates carried pLVPK-like virulence plasmid in this study, it is not economical to perform the phenotypic analysis for all the 46 isolates, especially the animal experiments (mouse sepsis model), so phenotypic analysis was only performed for 4 isolates. These 4 isolates were selected according to the different sizes of pLVPK-like virulence plasmids they carried (or the different missing plasmid fragment when compared to plasmid pLVPK). They reflect the virulence potential of polymyxin-resistant CRKP carrying pLVPK-like virulence plasmid of different sizes.

4. The authors found that the cause of polymyxin resistance in these CRKP isolates was mutation-mediated and not via horizontal transfer of MCR-1; could the authors add some further clarity/thoughts about:
a) Why these mutations first appeared in 2014 despite collection since 2003; were there any changes to clinical procedure that may have caused this.

Response: Polymyxins have been re-used widely for the treatment of CRKP from 2017 in clinical practice in China after abandoning its use in last century, which may facilitate the emergence of clinical polymyxin-resistant CRKP strains in China. So, the proportion of polymyxin-resistant CRKP strains increased significantly after 2017 (from 0.28% in 2017 to 2.08% in 2018).

b) Why did mutation rates increase over time, yet the proportion of strain backgrounds remain the same (except 2014 and 2017)?

Response: The increase in mutation rates of these polymyxin resistance related genes is attributed to the widespread use of polymyxins, which is not strictly related to the strain backgrounds.

5. Line 322: The strain 14-109 was used as a reference for core-SNV alignment and comparison with Snippy. This assembly (ASM3967506v1, GCA_039675065.1) is fragmented across 108 contigs and is therefore SNV information may be lost in genomic regions that frequently assemble poorly, such as the K-locus. What was the rationale behind using this strain as a reference when most strains are ST11? There are completed ST11 genomes available which would make a better reference.

Response: These strains have not undergone long-read sequencing, so we cannot obtain a complete sequence to be used as a reference.
The reason for selecting strain 14-109 as a reference is that it was collected relatively early (in 2014) among these 112 polymyxin-resistant CRKP strains. Although strain 14-109 is not an ST11 strain, the generated core genome alignment of 112 polymyxin-resistant CRKP strains is still approximately 5.1 Mbp, representing approximately 90.8% of the reference genome length (5.6 Mbp). The phylogenetic tree constructed based on the SNPs of this core genome alignment can fully represent the genetic relationship among our strain collection.

6. Line 323: Snippy produces a core-SNP alignment, but not a phylogenetic tree. What algorithm was used to produce the phylogenetic tree? Please list the software, version, algorithm and method used to root the tree.

Response: The core-SNP alignment of test strains was generated by Snippy v4.6, with assembled genome sequence of strain 14-109 as a reference. This alignment was then utilized to infer a phylogenetic tree using RAxML v8.2.12. The method has been revised accordingly.

Minor comments:
7. Line 30: “Totally 112 polymyxin-resistant CRKP isolates were identified.” Replace “Totally” with “In total,”

Response: Revised accordingly.

8. Line 296: How were the polymyxin-resistant CRKP isolates identified initially, this is not made clear.

Response: We have 4455 non-duplicate CRKP strains in our collection, then the polymyxin-resistant CRKP isolates were identified by determining the MIC of polymyxin B.

9. Line 319: K- and O-locus calls are reported so Kaptive needs to be cited (Wyres et al., MGen

2016) as per the Kleborate documentation (https://kleboratemodular.readthedocs.io/en/latest/#citations).

Response: The publication "Wyres, K. L., Wick, R. R., Gorrie, C., Jenney, A., Follador, R., Thomson, N. R., & Holt, K. E. (2016). Identification of Klebsiella capsule synthesis loci from whole genome data. Microbial genomics, 2(12), e000102." has been cited here.

10. Line 324: How were complete plasmid sequences determined from short-read draft assemblies?

Response: The sequence of the reference plasmid pLVPK in the outermost ring in Fig 4a is a complete plasmid sequence downloaded from NCBI database. After a BLASTn comparison with the reference plasmid, short-read draft assemblies from a test strain showed >99% identity and >80 coverage with reference plasmid pLVPK will be considered to carry a pLVPK-like virulence plasmid, since the backbone of these plasmids is relatively conserved and short-reads of a test strain can well align to the reference sequence with high coverage. Then, we use the complete sequence of plasmid pLVPK as the reference in the outermost ring, and use the short-read draft assemblies of test strains in the inner rings to generate the plasmid comparison figure by BLAST Ring Image Generator (BRIG).

11. Lines 249-251: There is conflicting evidence on whether polymyxin resistance results in increased enhanced virulence in Kp; the study cited here used a different lineage (ST258) which is not seen in this geography and the G. mellonella model is not able to distinguish between hv and classical Kp (Russo and MacDonald, mSphere, 2020). In addition, another study has shown that other polymyxin mutations (PhoQ) can sensitize Kp to IgM-mediated complement killing (van der Lans, S.P.A., et al., Sci Rep 2023).

Response: The sentence "It should be stressed that genetic alternations in the *mgrB* gene not only confer resistance to polymyxins, but also enhance the virulence level of *K. pneumoniae* in the *G. mellonella* waxworm infection model through reducing antimicrobial peptide susceptibility and impairing activation of early host defense response." has been deleted.

12. Table 2: Why were the strains not tested against colistin (Polymyxin E)?

Response: Polymyxin B is used more frequently than colistin for the treatment of CRKP infections in clinical practice in China.

13. Fig1: The resolution of this figure is too low making it difficult to read. Please increase the resolution of this figure.

Response: The resolution of Fig 1 has been increased.

14. Fig4a: The legend is recycled so multiple strains have the same key. This is bad practice for figures and make it impossible to interpret the data. One solution would be to cluster the plasmid contigs so only non-redundant sequences are compared, reducing the number of rings.

Response: The ring No. have been added to the legend and figure in Fig4a to make it easier to interpret the data. The ring colours are labelled from light blue to dark blue in every 10 rings to make it easier to differentiate.

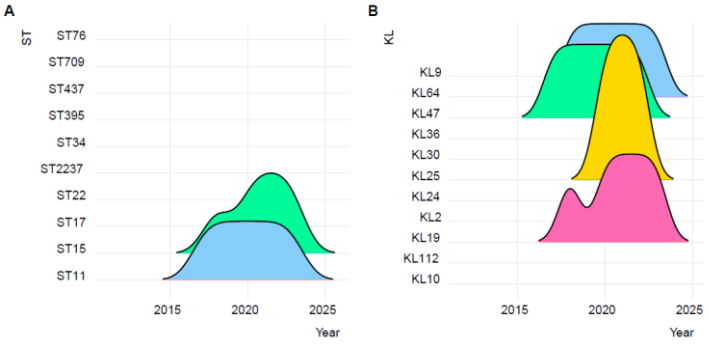
15. Fig4bcd: The legend is on the far right, so it is unclear that it refers to b and c too. The

legend should be in solid colours and put above all figures, so it is clear the key refers to all panels.

Response: The legend on the far right is only for Fig 4d. The legends (strain names) for Fig 4b and 4c are labelled under the X-axis, respectively. For Fig 4d, survival curves of FJ8, 19-70, 22-HN6 and 23-GX20 are same, so we use different colours to differentiate these four isolates.

16. It would be good to see a temporal breakdown of the clones over the sampling periods to demonstrate the proportion of clones over time. This can be represented as a ridge plot, with time on the X-axis and clone proportion on the Y-axis.

Response: We created ridge plots based on different ST (A) or KL (B) types as follows. However, the ridge plot only showed the two most common ST and four most common KL types, while other ST and KL types could not be shown in the ridge plots due to their small number of strains over time. So, we did not add the ridge plots into the manuscript.



Response to Review Comments

Reviewers' comments:

Reviewer #1 (Remarks to the Author):
Dear Author
Thank you for your manuscript submission. The manuscript is interesting; however, a revision is needed as below:

1. Please do read and add the following papers to References section to have fruitful Introduction and Discussion sections:

Emerging multidrug-resistant bacterial pathogens "superbugs": A rising public health threat. Front Microbiol. 2023 Feb 1;14:1135614. doi: 10.3389/fmicb.2023.1135614. PMID: 36819057; PMCID: PMC9930894.

Carbapenem-Resistant Klebsiella pneumoniae: Virulence Factors, Molecular Epidemiology and Latest Updates in Treatment Options. Antibiotics (Basel). 2023 Jan 21;12(2):234. doi: 10.3390/antibiotics12020234. PMID: 36830145; PMCID: PMC9952820.

Pan-Genome Plasticity and Virulence Factors: A Natural Treasure Trove for Acinetobacter baumannii. Antibiotics (Basel). 2024 Mar 14;13(3):257. doi: 10.3390/antibiotics13030257. PMID: 38534692; PMCID: PMC10967457.

Molecular Detection of gyrA Mutation in Clinical Strains of Klebsiella pneumoniae. Iran J Public Health. 2022 Oct;51(10):2334-2339. doi: 10.18502/ijph.v51i10.10992. PMID: 36415795; PMCID: PMC9647615.

Virulence factors, antibiotic resistance patterns, and molecular types of clinical isolates of Klebsiella Pneumoniae. Expert Rev Anti Infect Ther. 2022 Mar;20(3):463-472. doi: 10.1080/14787210.2022.1990040. Epub 2021 Oct 28. PMID: 34612762.

Metallo-ß-lactamases: a review. Mol Biol Rep. 2020 Aug;47(8):6281-6294. doi: 10.1007/s11033-020-05651-9. Epub 2020 Jul 11. PMID: 32654052.

Antimicrobial Agents and Urinary Tract Infections. Curr Pharm Des. 2019;25(12):1409-1423. doi: 10.2174/1381612825999190619130216. PMID: 31218955.

Prevalence of tetracycline resistance genes tet (A, B, C, 39) in Klebsiella pneumoniae isolated from Tehran, Iran. Iranian Journal of Medical Microbiology. 2022 Apr 10;16(2):141-7.

Response: We have read the above papers. Four papers have been cited in the Discussion and added in the Reference section.

2. Please do revise the Keywords as below:
Polymyxin resistance; carbapenem resistance; Klebsiella pneumoniae; clinical isolates; molecular epidemiology; phylogenetic analysis

Response: Revised accordingly.

3. Please do add the type of clinical samples: urine, blood, etc.

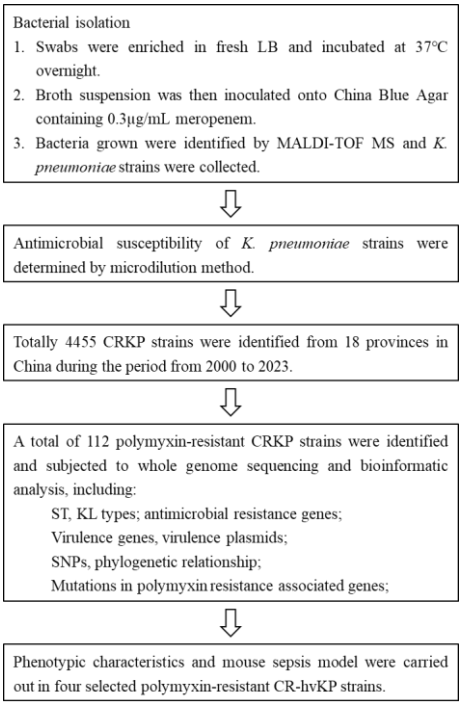
Response: Sorry, we cannot provide the type of clinical samples since many of them are missing.

4. Please do write the antibiotic sensitive test section in accordance with standard format: including the name of antibiotics (standard unit/ name of Company/Country)

Response: The name of antibiotics has been added. The sentence has been revised to "The susceptibility of polymyxin-resistant CRKP isolates to 15 commonly used antibiotics (including amikacin, aztreonam, cefepime, cefmetazole, cefoperazone-sulbactam, cefotaxime, ceftazidime, ceftazidime-avibactam, ciprofloxacin, ertapenem, meropenem, piperacillin-tazobactam, polymyxin B and tigecycline (Sigma-Aldrich, USA)) was determined using broth microdilution method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines."

5. Please do add a flow chart to show all the procedures done within the current study.

Response: A flow chart showing the procedures has been added in Supplementary Materials as follows.



Supplementary Fig S1. Flow chart diagram showing the isolation and analysis of polymyxin-resistant CRKP strains in this study.

6. It would be very interesting if the age range and the gender of the patients were included, too. I recommend to add these items to your study, too.

Response: Sorry, we cannot provide the age and gender of the patients, because according to the hospital's relevant regulations, patients' clinical information cannot be disclosed.

Reviewer #2 (Remarks to the Author):

Thank you to Xie et al., for submitting the work entitled: “Polymyxin resistance in clinical carbapenem-resistant Klebsiella pneumoniae strains in China: molecular epidemiological study from 2000~2023”

Here, the authors investigated the prevalence of polymyxin resistance in carbapenem-resistant Klebsiella pneumoniae (CRKP) strains in China and the underlying molecular mechanisms leading to resistance. Of the 4455 clinical CRKP strains collected, 112 were found to be polymyxin resistant, which underwent further molecular and phenotypic analysis. Following sequencing, draft assemblies of these strains were analysed with Kleborate to determine AMR and virulence gene content, and phylogenetic relationships were determined with Snippy. Six strains were further phenotypically characterised for hypermucoviscosity, capsule production and virulence in mice.

This is an impressive collection with accompanying sequence data and is a welcome resource to the community. However, quality of the data analysis is lacking, and some results are difficult to interpret if not completely omitted, therefore I suggest major revisions. Detailed comments are as follows:

Major comments:
1. There needs to be a supplementary table listing every strain, its relative NCBI accession, assembly statistics and other genotypic data (Kleborate, Kaptive, etc). This genotypic data is only summarised in text and must be provided.

Response: A supplementary table listing every strain, its NCBI accession, assembly statistics, and genotypic data is provided.

2. There are no methods sections on the mucoviscosity assay (Fig 4b), capsule production (Fig 4c) or mouse sepsis model (Fig 4d); these must be included in detail for reproducibility.

Response: The methods of mucoviscosity assay, capsule production and mouse sepsis model have been added to the Methods section.

3. Why were only 6 strains chosen for the phenotypic analysis? What was the rationale behind choosing these 6 strains? Do they truly reflect the diversity of the dataset of the polymyxin resistant CRKP in this study?

Response: Totally 46 polymyxin-resistant CRKP isolates carried pLVPK-like virulence plasmid in this study, it is not economical to perform the phenotypic analysis for all the 46 isolates, especially the animal experiments (mouse sepsis model), so phenotypic analysis was only performed for 4 isolates. These 4 isolates were selected according to the different sizes of pLVPK-like virulence plasmids they carried (or the different missing plasmid fragment when compared to plasmid pLVPK). They reflect the virulence potential of polymyxin-resistant CRKP carrying pLVPK-like virulence plasmid of different sizes.

4. The authors found that the cause of polymyxin resistance in these CRKP isolates was mutation-mediated and not via horizontal transfer of MCR-1; could the authors add some further clarity/thoughts about:
a) Why these mutations first appeared in 2014 despite collection since 2003; were there any changes to clinical procedure that may have caused this.

Response: Polymyxins have been re-used widely for the treatment of CRKP from 2017 in clinical practice in China after abandoning its use in last century, which may facilitate the emergence of clinical polymyxin-resistant CRKP strains in China. So, the proportion of polymyxin-resistant CRKP strains increased significantly after 2017 (from 0.28% in 2017 to 2.08% in 2018).

b) Why did mutation rates increase over time, yet the proportion of strain backgrounds remain the same (except 2014 and 2017)?

Response: The increase in mutation rates of these polymyxin resistance related genes is attributed to the widespread use of polymyxins, which is not strictly related to the strain backgrounds.

5. Line 322: The strain 14-109 was used as a reference for core-SNV alignment and comparison with Snippy. This assembly (ASM3967506v1, GCA_039675065.1) is fragmented across 108 contigs and is therefore SNV information may be lost in genomic regions that frequently assemble poorly, such as the K-locus. What was the rationale behind using this strain as a reference when most strains are ST11? There are completed ST11 genomes available which would make a better reference.

Response: These strains have not undergone long-read sequencing, so we cannot obtain a complete sequence to be used as a reference.
The reason for selecting strain 14-109 as a reference is that it was collected relatively early (in 2014) among these 112 polymyxin-resistant CRKP strains. Although strain 14-109 is not an ST11 strain, the generated core genome alignment of 112 polymyxin-resistant CRKP strains is still approximately 5.1 Mbp, representing approximately 90.8% of the reference genome length (5.6 Mbp). The phylogenetic tree constructed based on the SNPs of this core genome alignment can fully represent the genetic relationship among our strain collection.

6. Line 323: Snippy produces a core-SNP alignment, but not a phylogenetic tree. What algorithm was used to produce the phylogenetic tree? Please list the software, version, algorithm and method used to root the tree.

Response: The core-SNP alignment of test strains was generated by Snippy v4.6, with assembled genome sequence of strain 14-109 as a reference. This alignment was then utilized to infer a phylogenetic tree using RAxML v8.2.12. The method has been revised accordingly.

Minor comments:
7. Line 30: “Totally 112 polymyxin-resistant CRKP isolates were identified.” Replace “Totally” with “In total,”

Response: Revised accordingly.

8. Line 296: How were the polymyxin-resistant CRKP isolates identified initially, this is not made clear.

Response: We have 4455 non-duplicate CRKP strains in our collection, then the polymyxin-resistant CRKP isolates were identified by determining the MIC of polymyxin B.

9. Line 319: K- and O-locus calls are reported so Kaptive needs to be cited (Wyres et al., MGen

2016) as per the Kleborate documentation (https://kleboratemodular.readthedocs.io/en/latest/#citations).

Response: The publication "Wyres, K. L., Wick, R. R., Gorrie, C., Jenney, A., Follador, R., Thomson, N. R., & Holt, K. E. (2016). Identification of Klebsiella capsule synthesis loci from whole genome data. Microbial genomics, 2(12), e000102." has been cited here.

10. Line 324: How were complete plasmid sequences determined from short-read draft assemblies?

Response: The sequence of the reference plasmid pLVPK in the outermost ring in Fig 4a is a complete plasmid sequence downloaded from NCBI database. After a BLASTn comparison with the reference plasmid, short-read draft assemblies from a test strain showed >99% identity and >80 coverage with reference plasmid pLVPK will be considered to carry a pLVPK-like virulence plasmid, since the backbone of these plasmids is relatively conserved and short-reads of a test strain can well align to the reference sequence with high coverage. Then, we use the complete sequence of plasmid pLVPK as the reference in the outermost ring, and use the short-read draft assemblies of test strains in the inner rings to generate the plasmid comparison figure by BLAST Ring Image Generator (BRIG).

11. Lines 249-251: There is conflicting evidence on whether polymyxin resistance results in increased enhanced virulence in Kp; the study cited here used a different lineage (ST258) which is not seen in this geography and the G. mellonella model is not able to distinguish between hv and classical Kp (Russo and MacDonald, mSphere, 2020). In addition, another study has shown that other polymyxin mutations (PhoQ) can sensitize Kp to IgM-mediated complement killing (van der Lans, S.P.A., et al., Sci Rep 2023).

Response: The sentence "It should be stressed that genetic alternations in the *mgrB* gene not only confer resistance to polymyxins, but also enhance the virulence level of *K. pneumoniae* in the *G. mellonella* waxworm infection model through reducing antimicrobial peptide susceptibility and impairing activation of early host defense response." has been deleted.

12. Table 2: Why were the strains not tested against colistin (Polymyxin E)?

Response: Polymyxin B is used more frequently than colistin for the treatment of CRKP infections in clinical practice in China.

13. Fig1: The resolution of this figure is too low making it difficult to read. Please increase the resolution of this figure.

Response: The resolution of Fig 1 has been increased.

14. Fig4a: The legend is recycled so multiple strains have the same key. This is bad practice for figures and make it impossible to interpret the data. One solution would be to cluster the plasmid contigs so only non-redundant sequences are compared, reducing the number of rings.

Response: The ring No. have been added to the legend and figure in Fig4a to make it easier to interpret the data. The ring colours are labelled from light blue to dark blue in every 10 rings to make it easier to differentiate.

15. Fig4bcd: The legend is on the far right, so it is unclear that it refers to b and c too. The

legend should be in solid colours and put above all figures, so it is clear the key refers to all panels.

Response: The legend on the far right is only for Fig 4d. The legends (strain names) for Fig 4b and 4c are labelled under the X-axis, respectively. For Fig 4d, survival curves of FJ8, 19-70, 22-HN6 and 23-GX20 are same, so we use different colours to differentiate these four isolates.

16. It would be good to see a temporal breakdown of the clones over the sampling periods to demonstrate the proportion of clones over time. This can be represented as a ridge plot, with time on the X-axis and clone proportion on the Y-axis.

Response: We created ridge plots based on different ST (A) or KL (B) types as follows. However, the ridge plot only showed the two most common ST and four most common KL types, while other ST and KL types could not be shown in the ridge plots due to their small number of strains over time. So, we did not add the ridge plots into the manuscript.