nature portfolio

Peer Review File

Systematic surveillance of Carbapenemase-Producing Enterobacteriales reveals persistent spread of IMP-4 IncM2 plasmids in New Caledonia

Corresponding Author: Dr Julien Colot

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)
General comments
The authors draw an epidemiological picture of carbapenemase-producing Enterobacterales from New Caledonia collected over nine years. Whole genome sequencing was used to describe the clonal relation and to identify resistance genes. The collection includes larger outbreaks. The report is well written but is rather descriptive. I have doubts that this work meets the high standard of Communications Medicine.

Major concerns
Figure 1: the figure could be improved by reporting screening and clinical samples in relation to the overall samples that were taken. This could be done either by stacked bars or by adding the proportion of samples to each bar being positive for CPE. The increase of numbers can have two reasons: either transmission and dispersal or increased testing. That should be clarified.
Line 132: AST was only done for a subset of isolates (130/199). How was carbapenem-resistance detected if AST was not performed? This approach appears to be very uncommon and is not sound from my point of view. I strongly recommend doing AST for all isolates. One might assume that carbapenem-resistance could have been deduced from WGS but here again, not all but only a “representative” subset was subjected to WGS (89/199).
Line 350: It is well known that ESBL-plates are inappropriate to screen for OXA-48 due to the susceptibility of OXA-48 to third generation cephalosporins. The authors should therefore not claim that a CPE screening was done as long as proper CPE-specific screening plates are not used.

Minor issues
Abstract: please report the total n in brackets when showing proportions.
Line 95: revise the single bracket after “carbapenemase”.
Discussion: a section on limitations before the conclusions is missing.
Line 354: ceftolozane/tazobactam was tested but not reported.

Reviewer #2

(Remarks to the Author)
The manuscript ID COMMSMED-24-1363-T titled “Decade of Carbapenemase-Producing Enterobacterales in New Caledonia: Integrative Surveillance Though Genomic, Phenotypic and Clinical approaches” was submitted to Communications Medicine as an Article, to be considered for publication.

The study investigated the transmission patterns and genetic characteristics of CPE isolated in New Caledonia from 2013 to 2022. The authors studied 199 on duplicate CPE clinical isolates from 164 patients and 15 from hospital environmental surface. After phenotypic analysis with Vitek and genotypic study with Illumina of a selection of isolates, they found that most common genera were Enterobacter (34%) and Klebsiella (25%), with 194 isolates (98%) carrying IMP-type carbapenemase.

The dominant allele was blaIMP-4 associated by a bioinformatic tool with L/M plasmid. They performed outbreak analysis considering SNPs and Vitek MIC profiles and found 12 distinct outbreaks involving IMP producers.

The conception of the work is correct, the experimental design appropriate as well as the approach selected to perform the study. The work is original and relevant for the medical and scientific community. Still, this manuscript requires deep revision.
The complete manuscript should be revised by a native English speaker. There are incorrect terms, incorrect use of prepositions, comas, brackets, semicolons, etc
The major comment or observation besides the writing is that the authors should clearly define Outbreak. Throughout the text, it becomes confusing whether they are referring to an outbreak caused by a specific specie, or an outbreak caused by IMP-4 or an outbreak of an IncL/M plasmid. By looking at Figure 5, all large outbreaks were caused by IncL/M plasmids. It is not known the degree of similarity between them or the real conjugative capacity.

May be you should consider testing a representative selection of those isolates to perform a biparental conjugation assay, blast for identical Class 1 integrons to map you reads to confirm gene order, etc. It is important to provide additional evidence to you bioinformatic predictions. It is not clear whether the Class 1 integron harbouring blaIMP-4 is exactly the same in all the sequenced isolates. It is also not clear the complete gene sequence order detected in all Class 1 integrons.

Figure 4 and 5 are very well presented but they become a bit confusing considering excessive amount of information. Please consider revising the figures in terms of your own definition of Outbreak. You may be able to simplify them.

Once you define Outbreak, you may re order the explanation in result. Another comment regarding this is that in the discussion you discuss naming species and ST, but in results you only mention the “outbreaks”-. The Cluster chapter is hard to follow
In the discussion, the results are well contrasted with the existing bibliography.

Introduction
Mixed unrelated subjects (utility of Genomics and basic data about carbapenemase producing Enterobacterales)
Line 88, confusing with extra parenthesis. Please check the correct use of “ : “ they are usually used to provide a listing of things
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L95 extra)
L108 57 isolates, remove of

Reviewer #3

(Remarks to the Author)
In this retrospective molecular epidemiological investigation, Authors investigated the transmission patterns and genetic characteristics of CPE isolated in New Caledonia during the 2013-2022 period. Clinical, surveillance and environmental isolates were subjected to phenotypic (AST) and genotypic (WGS) characterization. Overall, results revealed the dominance of IMP-4 among collected CPE (e.g. Enterobacter, Klebsiella, Serratia), and pointed out to multispecies, oligoclonal transmission clusters likely fueled by a IncL/M-type plasmid. The topic is of interest, considering the paucity of data concerning the spread of CPE in NC, but the study suffers from some shortcomings that need to be addressed, thus providing the chance to improve the manuscript quality. My main concern is about the analysis of the IncL/M epidemic plasmid, most likely involved in spread of blaIMP-4 in the study setting. This point should be more thoroughly investigated (see comments below).

General comments
- it is unclear why AST was conducted just on 130 CPE isolates and not on the whole non-duplicate collection (n=199). On top of this, PIP/TAZO was evaluated just in 112 clinical CPE isolates. Please clarify.
- (l.151) please define if it was an IncL or IncM plasmid, and provide subtyping (10.1371/journal.pone.0123063), carefully checking if the same replicon type was hosted by all IMP-producing organisms. This will certainly be of help to corroborate the hypothesis of a “plasmid outbreak”, as seen with pOXA-48a elsewhere. To this end, a recent review showed that incM1 plasmids (among IncL/M) were associated with IMP in Asia Pacific regions (https://doi.org/10.1080/14787210.2024.2305854).
- please clarify if environmental isolates were clonally related (cgSNPs > 21) to clinical ones
- since present results highlight the value of studying plasmids, particularly during dissemination episodes involving different strains, and likely suggest a pivotal role of an incL/M (epidemic) plasmid in dissemination of blaIMP-4 in the study setting, long read sequencing of representative isolates (one per species?) should be performed to corroborate this hypothesis (proposed throughout the text). This will allow for a more comprehensive comparative analysis of IncL/M plasmids from NC, as well as with related replicons from areas suffering from high prevalence of IMP-4 (see for example doi: 10.1128/AAC.01189-12, doi: 10.1128/AAC.00588-16)
- on top of the previous comment, I will check for the status of the tir locus (encoding a transfer inhibition protein) located on the IncL/M plasmid backbones. The insertional inactivation of tir by Tn1999 has been associated with a higher conjugal transfer frequency of plasmid pOXA-48a, so that this genetic configuration has been deemed as a key factor contributing to the successful dissemination of pOXA-48a at a global scale (https://doi.org/10.1080/14787210.2024.2305854).
- It would be interesting to explore the conjugal transfer capabilities (i.e. frequency) of IncL/M plasmids from representative

(one per genus?) IMP-4 producing organisms. This would provide the chance to improve the manuscript quality, mainly based on descriptive analyses (WGS, AST), by adding a functional one.

Specific comments
l.147-149: not clear how LFIA confirmed production of a specific enzyme variant, here IMP-4, (vs IMP-like). Please clarify
l.150: any information about the integron structure/numbering (please refer to the Integrall database)?
Figure 4 is pretty hard to read/follow. Authors can try to physically split the a-b-c panels horizontally, by grouping according to bacterial species (thus avoiding "X").
l.174: cgSNP < 21 reported just in Serratia? What about other CPE and associated clones (STs)?
l.181: when reporting mean values, please state the proper error measurement (IQR)
l.184: resistant to what?
l.192: I would consider to better define "small outbreak" as transmission clusters due to the very low numbers of isolates involved.
l.267-274: respectfully disagree on that point. On paper, RLFP may prove useful in ruling out/in if a common plasmid backbone is detected across multiple isolates, reinforcing the suspect of an outbreak. However, Enterobacterales most often host multiple plasmids (2 to 5), which could provide some noisy background in RFLP and could prevent investigators from properly assessing a plasmid outbreak. The RLPF support is further limited in those cases where multiclonal, multispecies outbreaks are suspected.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Thank you for the revised version. The authors addressed almost all points of my report.
In line 468-470 it should be clearly stated how many of the archived isolates were recovered for AST.
The challenge to detect OXA-48 with ESBL-screening plates should be addressed as a limitation at the end of the discussion section. That is a relevant weakness and should be transparently communicated.

Reviewer #2

(Remarks to the Author)
The manuscript #COMMSMED-24-1363A, entitled "Integrative Surveillance Through Genomic, Phenotypic and Clinical Approaches of Carbapenemase-Producing Enterobacterales Reveals Persistent Spread of an IMP-4 IncM2 Plasmid in New Caledonia" was submitted to Communications Medicine to be considered for publication. This manuscript reports a decade-long (2013–2022) surveillance study on Carbapenemase-Producing Enterobacterales (CPE) in New Caledonia. The work integrates phenotypic, genomic, and epidemiological data to describe how blaIMP-4 primarily carried in IncM2 plasmids, has persisted and spread in the clinical and environmental settings of the hospital. The authors also detected four NDMs, two in E. coli, one in Providencia spp. and in one Providencia that was a double carbapenemase producer carrying IMP-4 and NMD-1.
The retrospective study originally included 199 clinical and 15 environmental samples, belonging to six Enterobacterales species, including Enterobacter and Klebsiella. Most of the isolates harbored IMP-4. Long-read sequencing identified seven IncM2 plasmid variants. The study highlights 12 transmission events, 10 linked to IMP-4 IncM2 plasmids.
The manuscript has regional value given the scarcity of antimicrobial resistance (AMR) surveillance studies in Pacific Island territories. Moreover, the findings differ from those of other geographical regions were NDM and KPC are the major carbapenemases detected. Therefore, this work adds knowledge to the field of study however, its potential impact is reduced by issues of English writing, organization, result presentation, and inconsistent terminology, among other issues.
Major comments
The Results section combines phenotypic, genomic, and epidemiological data without a clear logical progression. For example, Section 2 (lines 129–202) alternates between isolate distribution, antimicrobial susceptibility testing, and plasmid characterization, which makes it difficult to follow the narrative. It would be important to clarify the criteria used to select the 89 representative isolates for sequencing. Were these chosen based on diversity, year of isolation, or suspicion of outbreak involvement? In addition, please provide a clear definition of what the authors consider an outbreak.
Another point that requires clarification is the rationale for the choice of antimicrobial agents tested, given that most isolates are Enterobacterales carrying metallo-β-lactamases. Specifically, why was piperacillin-tazobactam (PTZ) retested using the Etest? This compound is not typically relevant for the detection of blaIMP or other carbapenemases.
Some findings appear rare or, to the best of my knowledge, not previously described, such as the localization of blaNDM within a class 1 integron on an IncC plasmid. This observation should be confirmed either by manual curation (including identification of the attC site and cassette position of blaNDM) or by another reliable approach. The association of IncC plasmids with blaNDM is well established; blaNDM is usually found within a truncated Tn125-derived transposon, followed by conserved genes such as bleMBL, trpF, dsbD, cutA, groS, and groL. Variants exist (e.g., bleMBL, dap, ampR, hybF, sul1), but the presence of blaNDM as a gene cassette within a class 1 integron is unique. Therefore, I strongly recommend performing manual curation and addressing this unusual finding in the Discussion section.
Figures 3 and 4 contain an excess of information, which makes them difficult to interpret. The authors are encouraged to highlight only the most relevant data and transfer dense or secondary elements to the supplementary material. Creating species-specific phylogenetic trees would help clarify transmission events.

The description of spread events is also limited and confusing. For instance, the branch containing all Citrobacter ST98 isolates appears phylogenetically identical—please comment on this observation. Furthermore, how were transmission events verified following hygiene investigations? Please describe this process in more detail.
In Serratia, two potential clusters seem to be present (one with 6 isolates and another with 13), composed of closely related strains but belonging to three different Serratia species according to Figure 4. This discrepancy needs clarification. I suggest generating separate phylogenetic trees for species involved in transmission events to improve resolution. Finally, in the tree, the “c” and “d” columns appear redundant, presenting similar information with different labels—please review and clarify this.
Minor comments
Line 140. an OXA-48 was mentioned (CLIN189), but then it was not shown in the phylogenetic tree, nor in Figure 4.
L166 it is not clear if 15 CPE with incC were found in this work or in a report by others
L77 OXA-48 enzymes are also serine enzymes
L114-117 please list items in decreasing order
L131. Considering you had access to a MaldiTOF and then to WGS, please indicate the correct genus and species from the beginning.
L176-179 please re write this sentence. It is difficult to understand
L216 The conjugation assay was not positive in the conditions tested. Please clarify this point. How are transconjugants confirmed? PCR?
L222-224 What was the criteria used to define outbreak? What species? PFGE?
L456-457 carbapenem resistance was assessed with ertapenem (a carbapenem) and then temocillin and Ceftolozane/tazobactam… please explain the rationale of using these drugs to determine carbapenem resistance
L481 what antimicrobial did you analyze to determine spread event and how?
L534 where is the figure or results corresponding to these methods?
L540 please define the criteria chosen to determine what plasmids were going to be sequenced.

Version 2:

Reviewer comments:

Reviewer #2

(Remarks to the Author)
I would like to thank the authors for the work done to improve the quality of the manuscritpt #COMMSMED-24-1363A, entitled “Integrative Surveillance Through Genomic, Phenotypic and Clinical Approaches of Carbapenemase-Producing Enterobacterales Reveals Persistent Spread of an IMP-4 IncM2 Plasmid in New Caledonia”

All major comments have been addressed accordingly. Still, there are still several issues that should be attended described below.

Throughout the manuscript, Enterobacterales was written without italics (DOI 10.1099/ijsem.0.001485), some bla genes were written without the subscritp gene name (please capitalized and subscritp). Make sure results are explained in past tense (check the abstract and results section). AMR is either spread or disseminated, not diffused. The term diffusion was used many times (line 263, 531, 561 and so on). Please check all the legends to the figures. In some, (a) and (b) are missing.
Line 455-458, truncated structure of what? the conjugation process is directed by operon coded genes. Please read bibliography about the conjugation process and re write your conclusions without especulating why it was negative (doi: 10.1128/MMBR.00020-10, doi: 10.1093/nar/gkac1079, etc).

Reviewers comments

Reviewer #1 (Remarks to the Author): Referee #1: clinical microbiologist

General comments
The authors draw an epidemiological picture of carbapenemase-producing Enterobacterales from New Caledonia collected over nine years. Whole genome sequencing was used to describe the clonal relation and to identify resistance genes. The collection includes larger outbreaks. The report is well written but is rather descriptive. I have doubts that this work meets the high standard of Communications Medicine.

We added long reads sequencing on IncM2 plasmids and also conjugation experiments to add a functional part.

Major concerns
Figure 1: the figure could be improved by reporting screening and clinical samples in relation to the overall samples that were taken. This could be done either by stacked bars or by adding the proportion of samples to each bar being positive for CPE. The increase of numbers can have two reasons: either transmission and dispersal or increased testing. That should be clarified.

We added the total number of screenings to Figure 1a and b. We can therefore see that the increase is linked to transmission and spread, rather than an increase in the number of tests.

Line 132: AST was only done for a subset of isolates (130/199). How was carbapenem-resistance detected if AST was not performed? This approach appears to be very uncommon and is not sound from my point of view. I strongly recommend doing AST for all isolates. One might assume that carbapenem-resistance could have been deduced from WGS but here again, not all but only a "representative" subset was subjected to WGS (89/199).

The ASTs were not systematically performed on strains isolated from screening or environmental samples. For these isolates, carbapenem resistance was identified using synergy tests followed by an immunochromatographic test. For this study, we attempted to re-examine all strains, but unfortunately some had not been stored at -80°C. See Lines 468-470.

Line 350: It is well known that ESBL-plates are inappropriate to screen for OXA-48 due to the susceptibility of OXA-48 to third generation cephalosporins. The authors should therefore not claim that a CPE screening was done as long as proper CPE-specific screening plates are not used.

Although our screening method (ESBL agar) may have underestimated pure OXA-48-producing strains (without ESBL associated), their low reported prevalence in France and in our region (dominance of IMP-4) suggests a limited impact on our results. This limitation will be addressed in future studies through the systematic use of specific OXA-48 media since January 2025. Since its implementation, no OXA-48 strains have

been detected.

Minor issues
Abstract: please report the total n in brackets when showing proportions. **Done**
Line 95: revise the single bracket after "carbapenemase". **Done**
Discussion: a section on limitations before the conclusions is missing. **Done, see lines 396**
Line 354: ceftolozane/tazobactam was tested but not reported. Done **See Line 142.**

Referee #2: mechanisms of resistance in bacteria, including genomic epidemiology

The manuscript ID COMMSMED-24-1363-T titled "Decade of Carbapenemase-Producing Enterobacterales in New Caledonia: Integrative Surveillance Though Genomic, Phenotypic and Clinical approaches" was submitted to Communications Medicine as an Article, to be considered for publication.

The study investigated the transmission patterns and genetic characteristics of CPE isolated in New Caledonia from 2013 to 2022. The authors studied 199 on duplicate CPE clinical isolates from 164 patients and 15 from hospital environmental surface. After phenotypic analysis with Vitek and genotypic study with Illumina of a selection of isolates, they found that most common genera were Enterobacter (34%) and Klebsiella (25%), with 194 isolates (98%) carrying IMP-type carbapenemase. The dominant allele was blaIMP-4 associated by a bioinformatic tool with L/M plasmid. They performed outbreak analysis considering SNPs and Vitek MIC profiles and found 12 distinct outbreaks involving IMP producers.

The conception of the work is correct, the experimental design appropriate as well as the approach selected to perform the study. The work is original and relevant for the medical and scientific community. Still, this manuscript requires deep revision.
The complete manuscript should be revised by a native English speaker. There are incorrect terms, incorrect use of prepositions, comas, brackets, semicolons, etc

Manuscript has been revised by English speaker.

The major comment or observation besides the writing is that the authors should clearly define Outbreak. Throughout the text, it becomes confusing whether they are referring to an outbreak caused by a specific specie, or an outbreak caused by IMP-4 or an outbreak of an IncL/M plasmid. By looking at Figure 5, all large outbreaks were caused by IncL/M plasmids.

We revised our definition of the term outbreak. See Lines 485-491. We also revised the entire manuscript to ultimately refer to the spread and persistence of an IncM2-type plasmid in New Caledonia.

It is not known the degree of similarity between them or the real conjugative capacity.

We sequenced the representative plasmids in long reads, then mapping all short reads and we were able to produce Figure 5 and Figure 6, which shows the degree of similarity (MASH distance) between the plasmids. We were also able to conduct conjugation experiments, as shown in Figure 7 and Table S3.

May be you should consider testing a representative selection of those isolates to perform a biparental conjugation assay, blast for identical Class 1 integrons to map you reads to confirm gene order, etc. It is important to provide additional evidence to you bioinformatic predictions. It is not clear whether the Class 1 integron harbouring blaIMP-4 is exactly the same in all the sequenced isolates. It is also not clear the complete gene sequence order detected in all Class 1 integrons.

We agree with this suggestion and as you will see Figures 5 et 6.

Figure 4 and 5 are very well presented but they become a bit confusing considering excessive amount of information. Please consider revising the figures in terms of your own definition of Outbreak. You may be able to simplify them.

We agree with this suggestion Figure 4 is now simplified and we decided to delete former Figure 5.

Once you define Outbreak, you may re order the explanation in result. Another comment regarding this is that in the discussion you discuss naming species and ST, but in results you only mention the “outbreaks”-. The Cluster chapter is hard to follow
In the discussion, the results are well contrasted with the existing bibliography.

We have revised the entire manuscript and hope that it is now clearer.

Introduction
Mixed unrelated subjects (utility of Genomics and basic data about carbapenemase producing Enterobacterales)
Line 88, confusing with extra parenthesis. Please check the correct use of “ : “ they are usually used to provide a listing of things
L92 “…there are links notably with Australia” , please correct the sentence
L95 extra)
L108 57 isolates, remove of

The introduction has been rewritten taking these comments into account.

Referee #3: clinical microbiologist, antimicrobial resistance, resistance mechanisms

In this retrospective molecular epidemiological investigation, Authors investigated the transmission patterns and genetic characteristics of CPE isolated in New Caledonia during the 2013-2022 period. Clinical, surveillance and environmental isolates were subjected to phenotypic (AST) and genotypic (WGS) characterization. Overall, results revealed the dominance of IMP-4 among collected CPE (e.g. Enterobacter, Klebsiella, Serratia), and pointed out to multispecies, oligoclonal transmission clusters likely fueled by a IncL/M-type plasmid. The topic is of interest, considering the paucity of data concerning the spread of CPE in NC, but the study suffers from some shortcomings that need to be addressed, thus providing the chance to improve the manuscript quality. My main concern is about the analysis of the IncL/M epidemic plasmid, most likely involved in spread of blaIMP-4 in the

study setting. This point should be more thoroughly investigated (see comments below).

General comments
- it is unclear why AST was conducted just on 130 CPE isolates and not on the whole non-duplicate collection (n=199). On top of this, PIP/TAZO was evaluated just in 112 clinical CPE isolates. Please clarify.

The ASTs were not systematically performed on strains isolated from screening or environmental samples. For these isolates, carbapenem resistance was identified using synergy tests followed by an immunochromatographic test. Concerning PIP/TAZO, it is not an antibiotic that is systematically tested. For this study, we attempted to re-examine all strains, but unfortunately some had not been stored at -80°C. See lines 468-470.

- (l.151) please define if it was an IncL or IncM plasmid, and provide subtyping (10.1371/journal.pone.0123063), carefully checking if the same replicon type was hosted by all IMP-producing organisms. This will certainly be of help to corroborate the hypothesis of a "plasmid outbreak", as seen with pOXA-48a elsewhere. To this end, a recent review showed that incM1 plasmids (among IncL/M) were associated with IMP in Asia Pacific regions (https://doi.org/10.1080/14787210.2024.2305854).

Thank you for this comment. We have therefore typed our plasmids more precisely, and it is an IncM2-type plasmid See Lines 161-162.

- please clarify if environmental isolates were clonally related (cgSNPs > 21) to clinical ones

CLIN37 which was isolated on an environmental surface is related to clinical isolates as shown in Figure 4. We emphasize in the revised manuscript that the persistence of this strain in the environment allows us to link the 2017 and 2018 outbreaks in the ICU. See Lines 237-238.

CLINX isolated on environmental surface are related to clinical ones (CLIN80 and CLIN96), see Figure 4

CLIN59 isolated on environmental surface during outbreak in NICU are related to clinical ones, see Figure 4

CLIN122 related to CLIN115, see Figure 4.

Please see lines 368-395 for importance of environmental reservoirs

- since present results highlight the value of studying plasmids, particularly during dissemination episodes involving different strains, and likely suggest a pivotal role of an incL/M (epidemic) plasmid in dissemination of blaIMP-4 in the study setting, long read sequencing of representative isolates (one per species?) should be performed to corroborate this hypothesis (proposed throughout the text). This will allow for a more comprehensive comparative analysis of IncL/M plasmids from NC, as well as with related replicons from

areas suffering from high prevalence of IMP-4 (see for example doi: 10.1128/AAC.01189-12, doi: 10.1128/AAC.00588-16)

We agree with this suggestion and as you will see Figure 4.

- on top of the previous comment, I will check for the status of the tir locus (encoding a transfer inhibition protein) located on the IncL/M plasmid backbones. The insertional inactivation of tir by Tn1999 has been associated with a higher conjugal transfer frequency of plasmid pOXA-48a, so that this genetic configuration has been deemed as a key factor contributing to the successful dissemination of pOXA-48a at a global scale (https://doi.org/10.1080/14787210.2024.2305854).

We finally have an IncM2, and we have not found this same mechanism for IncM2 in the literature. However, it is interesting to note the drastic drop in conjugation frequency for the IncM2 plasmid carried by CLIN08. There are certainly elements missing that are necessary for conjugation.

- It would be interesting to explore the conjugal transfer capabilities (i.e. frequency) of IncL/M plasmids from representative (one per genus?) IMP-4 producing organisms. This would provide the chance to improve the manuscript quality, mainly based on descriptive analyses (WGS, AST), by adding a functional one.

We agree with this suggestion and as you will see Figure 7.

Specific comments

l.147-149: not clear how LFIA confirmed production of a specific enzyme variant, here IMP-4, (vs IMP-like). Please clarify

We agree with this suggestion and as you will see Lines 134.

l.150: any information about the integron structure/numbering (please refer to the Integrall database)?

We agree with this suggestion and as you will see Figure 5.

Figure 4 is pretty hard to read/follow. Authors can try to physically split the a-b-c panels horizontally, by grouping according to bacterial species (thus avoiding “X”).

While it is true that the figure can be difficult to read, it enables us to present a great deal of information, including species-based clustering based on the core genome (SNP), resistance gene content, the position of these genes on chromosomes or plasmids, associated AST and the suspected or confirmed involvement in a spread event. Compared to the previous version, we have integrated all sequenced strains and removed plasmid content.

l.174: cgSNP < 21 reported just in Serratia? What about other CPE and associated clones (STs)?

We added other links. Please see Lines 176-191.

l.181: when reporting mean values, please state the proper error measurement (IQR)

For this type of data, we find it more useful to give the minimum and maximum values. This allows us to see the link between the closest and most distant strains within a ST.

l.184: resistant to what?

Modified

l.192: I would consider to better define “small outbreak” as transmission clusters due to the very low numbers of isolates involved.

We agree with this suggestion and as you will see lines 485-491.

l.267-274: respectfully disagree on that point. On paper, RLFP may prove useful in ruling out/in if a common plasmid backbone is detected across multiple isolates, reinforcing the suspect of an outbreak. However, Enterobacterales most often host multiple plasmids (2 to 5), which could provide some noisy background in RFLP and could prevent investigators from properly assessing a plasmid outbreak. The RLPF support is further limited in those cases where multiclonal, multispecies outbreaks are suspected.

We agree with this argument and have removed this part from the discussion.

Reviewer #1 (Remarks to the Author):

Thank you for the revised version. The authors addressed almost all points of my report.

In line 468-470 it should be clearly stated how many of the archived isolates were recovered for AST.

We recovered 69 archived isolates for AST corresponding to strains from colonized patients. For isolates from infectious sample (n=50), ASTs were performed as part of the diagnosis. We add this information in the marked-up manuscript at line 181 and at line 175 in the revised manuscript.

The challenge to detect OXA-48 with ESBL-screening plates should be addressed as a limitation at the end of the discussion section. That is a relevant weakness and should be transparently communicated.

Thank you for your comment, we completely agree with this limitation. We added several lines concerning this weakness in the marked-up manuscript at lines 648-653 and at lines 612-616 in the revised manuscript.

Reviewer #2 (Remarks to the Author):

The manuscript #COMMSMED-24-1363A, entitled "Integrative Surveillance Through Genomic, Phenotypic and Clinical Approaches of Carbapenemase-Producing Enterobacterales Reveals Persistent Spread of an IMP-4 IncM2 Plasmid in New Caledonia" was submitted to Communications Medicine to be considered for publication. This manuscript reports a decade-long (2013–2022) surveillance study on Carbapenemase-Producing Enterobacterales (CPE) in New Caledonia. The work integrates phenotypic, genomic, and epidemiological data to describe how blaIMP-4 primarily carried in IncM2 plasmids, has persisted and spread in the clinical and environmental settings of the hospital. The authors also detected four NDMs, two in E. coli, one in Providencia spp. and in one Providencia that was a double carbapenemase producer carrying IMP-4 and NMD-1. The retrospective study originally included 199 clinical and 15 environmental samples, belonging to six Enterobacterales species, including Enterobacter and Klebsiella. Most of the isolates harbored IMP-4. Long-read sequencing identified seven IncM2 plasmid variants. The study highlights 12 transmission events, 10 linked to IMP-4 IncM2 plasmids. The manuscript has regional value given the scarcity of antimicrobial resistance (AMR) surveillance studies in Pacific Island territories. Moreover, the findings differ from those of other geographical regions were NDM and KPC are the major carbapenemases detected. Therefore, this work adds knowledge to the field of study however, its potential impact is reduced by issues of English writing, organization, result presentation, and inconsistent terminology, among other issues.

Thank you for these comments, concerning English writing, the revised manuscript has been revised by an English speaker. Thank to your comments we hope to have improved results presentation, and inconsistent terminology and answered your questions.

Major comments

The Results section combines phenotypic, genomic, and epidemiological data without a clear logical progression. For example, Section 2 (lines 129–202) alternates between isolate

distribution, antimicrobial susceptibility testing, and plasmid characterization, which makes it difficult to follow the narrative.

Thank you for your comment, we have rewritten this section, and we propose to add some subtitles in order to guide the logical flow. Please see lines 342-432 in the marked-up manuscript. Please see lines 332-395 in the revised manuscript.

It would be important to clarify the criteria used to select the 89 representative isolates for sequencing. Were these chosen based on diversity, year of isolation, or suspicion of outbreak involvement?

Regarding clinical isolates, yes, they were first selected based on their year of isolation in order to inform historical data. For example, we were able to sequence all those isolated between 2013 (first CPE isolated in NC) and 2015. Given the growing number of CPE found and the primary objective of this study, which was to work on hospital diffusion events, we then decided to sequence all those that were involved or suspected of being involved in spread events. Still with the aim of focusing on spread events, we also decided to sequence the environmental strains found during these epidemic episodes. We added information please see Lines 215-221 int the marked-up manuscript and lines 207-2014 in the revised manuscript.

In addition, please provide a clear definition of what the authors consider an outbreak.

From your initial comment on the previous version of the paper, we acknowledge that this may not be very clear. What we want to emphasize in our paper is that ultimately there may be only one epidemic, that of the IncM2 plasmid, as you noted (shifting the paradigm of a bacterial clone outbreak to a plasmid-supported outbreak). This paper therefore highlights that there have been several episodes at different times in different departments involving different bacteria, but that ultimately there may be only one outbreak involving the spread and persistence of the IncM2 plasmid carrying IMP-4. We have rewritten the revised manuscript taking this comment into account and hope this clarifies the message we aimed to convey. Please see Lines 206-210 and 531-532 in the marked-up manuscript and lines 197-205 and 493-497 in the revised manuscript.

Another point that requires clarification is the rationale for the choice of antimicrobial agents tested, given that most isolates are Enterobacterales carrying metallo-β-lactamases.

We tested the antibiotics included in the VITEK cards according to the year and type of infection, as these parameters may have varied over time. For strains isolated before 2015, the N-234 card was used. Between 2016 and August 2024, the N-372 card was used for urine-derived strains, while the N-234 card was used for all other isolates. Finally, for antimicrobial susceptibility testing (AST) performed retrospectively after August 2024, the N-441 card was used. We add information, please see Lines 170-175 in the marked-up manuscript and lines 167-171 in the revised manuscript.

Specifically, why was piperacillin-tazobactam (PTZ) retested using the Etest? This compound is not typically relevant for the detection of blaIMP or other carbapenemases.

PTZ was retested in case of unspected results: in our case, PTZ sensitivity for CPEs. We therefore retested using E-tests, the reference method.

Some findings appear rare or, to the best of my knowledge, not previously described, such as the localization of blaNDM within a class 1 integron on an IncC plasmid. This observation should be confirmed either by manual curation (including identification of the attC site and cassette position of blaNDM) or by another reliable approach. The association of IncC plasmids with blaNDM is well established; blaNDM is usually found within a truncated Tn125-derived transposon, followed by conserved genes such as bleMBL, trpF, dsbD, cutA, groS, and groL. Variants exist (e.g., bleMBL, dap, ampR, hybF, sul1), but the presence of blaNDM as a gene cassette within a class 1 integron is unique. Therefore, I strongly recommend performing manual curation and addressing this unusual finding in the Discussion section.

Thank you for your vigilance. As this is not the most important finding in this paper, given the low prevalence of this carbapenemase in New Caledonia, we did not investigate it further. After verification, this is indeed an error in the manuscript. Our data do not allow us to confirm that *bla*$_{NDM-1}$ is carried by a class 1 integron as reported in Table 3. We did find a class 1 integron in the isolates, but it carries other antibiotic resistance genes. For instance, the attc region was not reported on the same contig as *bla*$_{NDM-1}$. We are unable to perform long reads, but by blasting the contigs carrying the *bla*$_{NDM-1}$ gene, we see that they are not on a class 1 integron on the closest plasmids (https://ccb-microbe.cs.uni-saarland.de/plsdb2025/plasmid?NUCCORE_ACC=NZ_CP030720.1). They are potentially on a truncated Tn125-derived transposon, followed by conserved genes such as bleMBL, in our example. Based on our data, we can only state that *bla*$_{NDM-1}$ is predicted on InC plasmids for both *Shigella* and on an undefined incompatibility type plasmid (AA855 MOB_typer primary cluster). We have withdrawn class 1 integron statement in the manuscript. Please see Lines 415-418 in the marked-up manuscript and lines 392-395 in the revised manuscript. Thank you again for your insight and vigilance.

Figures 3 and 4 contain an excess of information, which makes them difficult to interpret. The authors are encouraged to highlight only the most relevant data and transfer dense or secondary elements to the supplementary material. Creating species-specific phylogenetic trees would help clarify transmission events.

Yes based on reviewers comments we decided to simplify the figure 4 in order to keep only relationships between isolates, ARG position and implication in spread events. The reader can find the association between transmission events and strains before genomic analysis in Figure 3 and the association between transmission events and strains using genomic data in Figure 4. For example, for *K. pneumoniae* ST307, without genomic data, the strains were not identified as being involved in a spread event (Metadata in Figure 3), but with genomics and hygiene investigations, a transmission cluster was identified (Figure 4). We also decided to split the phylogenetic tree in 5 separate trees focusing on each genus. We decided to do not phylogenetic tree for *Providencia* isolates because only two isolates were recovered.

The description of spread events is also limited and confusing. For instance, the branch containing all Citrobacter ST98 isolates appears phylogenetically identical—please comment on this observation.

Thank you for your comment, thank to our new Figure 3, we added several lines about relationship between isolates in each genus. We also added information about cgSNP. Please see lines 374-383 in the marked-up manuscript and lines 362-370 in the revised manuscript.

Also regarding spread events, we have grouped everything together in a single paragraph., please see lines 465 to 499 in the marked-up manuscript and lines 428-462 in the revised manuscript.

Furthermore, how were transmission events verified following hygiene investigations? Please describe this process in more detail.

When an CPE is detected in a healthcare department, all contact patients, i.e. those who have been hospitalized in the same department or who have shared the same healthcare team, are systematically screened (two screenings are performed 72 hours apart: culture then PCR). Contact positive cases are considered secondary cases and linked to the same episode. New hygiene investigations have taken up events that appear to be linked via genomic analysis. In the event of hospitalization in the same department with an interval of 1 month, cases are considered to be linked with spread event. Other strains or plasmids were considered presumed transmission links if patients had stayed in different departments but within the same one-month interval, or in the same department but with stays more than one month apart. We added this information please see Lines 202-210 in the marked-up manuscript and lines 193-199 in the revised manuscript.

In Serratia, two potential clusters seem to be present (one with 6 isolates and another with 13), composed of closely related strains but belonging to three different Serratia species according to Figure 4. This discrepancy needs clarification. I suggest generating separate phylogenetic trees for species involved in transmission events to improve resolution.

Thanks to the new Figure 3, we have added new elements. The TYGS approach suggests that some *Serratia* isolates are potentially a novel specie. ($dDDH_4 < 70\%$). Based on these results and new samples to be collected, we will investigate these isolates in more detail to see if they are indeed a new species or subspecies within the *S. marcescens* complex. We will also continue to monitor *Serratia sarumanii* following the work of Klages et al. (2025). We added information in lines 368-372 in the marked-up manuscript and lines 360-365 in the revised manuscript.

Finally, in the tree, the “c” and “d” columns appear redundant, presenting similar information with different labels—please review and clarify this.

We prefer to keep them because some strains are not involved in spread events, so it is important that the service in which the strains were found also appears.

Minor comments

Line 140. an OXA-48 was mentioned (CLIN189), but then it was not shown in the phylogenetic tree, nor in Figure 4.

Unfortunately, we were unable to sequence CLIN189. The isolate have not been conserved.

L166 it is not clear if 15 CPE with incC were found in this work or in a report by others

The 15 CPE were found in this work. We remove “also” in the sentence. Please see Line 406 in the marked-up manuscript and line 387 in the revised manuscript.

L77 OXA-48 enzymes are also serine enzymes

Thank you for this comment, we modified the sentence. Please see line 102 in the marked-up manuscript and line 102 in the revised manuscript.

L114-117 please list items in decreasing order

Done, please see lines 327-328 in the marked-up manuscript and lines 315-319 revised manuscript.

L131. Considering you had access to a MaldiTOF and then to WGS, please indicate the correct genus and species from the beginning.

This concerns data for 199 CPEs for which we only have MALDI-ToF identification data and for which we wish to rely solely on genus. We only have the correct species for the 89 sequenced strains.

L176-179 please re write this sentence. It is difficult to understand

Following, Figure 4 simplification, we deleted the sentence. Please see lines 417-420 in the marked-up manuscript.

L216 The conjugation assay was not positive in the conditions tested. Please clarify this point. How are transconjugants confirmed? PCR?

We appreciate your feedback and would like to clarify the apparent discrepancy between the reported conjugation failure and the data presented in Figure 7.

As detailed in Table S2, the conjugation assay for the combination CLIN08 + CIP104049 did not yield a detectable transconjugant. However, following the methodology described in Fu et al. (2025), we initially included an arbitrary value of 9 CFU/mL (corresponding to the limit of detection) to represent this negative result. To prevent misinterpretation, we propose to remove this data point from Figure 7.

If your comment pertains to the underlying reasons for this negative result, we can propose the following hypothesis: The CLIN08 variant exhibits a highly truncated structure (as shown in Figure 5), which may impair key conjugation machinery. This is further supported by its significantly lower conjugation frequency with the AE strain compared to other variants, suggesting that essential transfer elements may be missing or non-functional. We added several lines in the marked-up manuscript (Lines 456-459) and lines 420-422 in the revised manuscript.

We hope this clarification resolves any ambiguity. Please let us know if further details would be helpful.

For transconjugants obtained, they are confirmed with IncM2 qPCR (please see Lines 315-317 in the marked-up manuscript and lines 305-307 in the revised manuscript,) and AST profile (please see Table S2)

L222-224 What was the criteria used to define outbreak? What species? PFGE?

Suspected spread event were characterised by the incidental discovery of the same CPE based on (i) species identification (using MALDI-ToF), AST (when available), (ii) type of carbapenemase (using NG-CARBA-5® immunochrmatographic assays) leading to the detection of a secondary case, or the detection of same CPE in the same department at short interval (less than one month). When an CPE is detected in a healthcare department, all contact patients, i.e. those who have been hospitalized in the same department or who have shared the same healthcare team, are systematically screened (two screenings are performed 72 hours apart: culture then PCR). Contact positive cases are considered secondary cases and linked to the same episode. In this study, a spread event

was considered as an outbreak if there were at least five secondary cases, otherwise, if there are fewer than 5 secondary cases, we considered this event as transmission cluster. Please see lines 197-210 in the marked-up manuscript and lines 187-205 in the revised manuscript.

L456-457 carbapenem resistance was assessed with ertapenem (a carbapenem) and then temocillin and Ceftolozane/tazobactam… please explain the rationale of using these drugs to determine carbapenem resistance

The phenotypic screening algorithm for carbapenemase-producing Enterobacterales is used: recommendations from CA-SFM/EUCAST. There was a first version from 2015 to 2021 (tested molecules: ertapenem, ticarcillin-clavulanic acid, temocillin) then a new version in 2022 (tested molecules: meropenem or imipenem, ceftazidim-avibactam, temocillin). According to CPE epidemiology in NC, in 2020, we adapted the 2015 version by adding ceftolozan-tazobactam (we have amoxicillin + clavulanic acid instead of ticarcillin+ clavulanic acid) to better detect IMPs. We have kept the same molecules since 2020 (ertapenem, ceftolazan tazobactam, temocillin). Please see lines 161-166 in the marked-up manuscript and lines 158-163 in the revised manuscript.

L481 what antimicrobial did you analyze to determine spread event and how?

Considering the random errors of the VITEK and clonal relationships based on WGS, we considered identical ASTs if they differed by no more than one result. Please see Lines 195-196 in the marked-up manuscript and lines 187-190 in the revised manuscript.

L534 where is the figure or results corresponding to these methods?

Results are reported in Fig 4 and we added cgSNP data in Lines 368 to 385 in the marked-up manuscript and lines 363-372 in the revised manuscript. All results are reported in Table S1.

L540 please define the criteria chosen to determine what plasmids were going to be sequenced.

We first mapped all the short reads to the reference sequence JX101693. This approach allowed us to classify IncM2 plasmids by variant. We then chose one representative of each variant (based on its species or involvement in specific transmission events, with the aim of making the best use of these long reads) for long read sequencing. We added information in the revised manuscript. Please see Lines 266-269 in the marked-up manuscript and lines 256-258 in the revised manuscript.

Reviewer #1 checked revision on behalf of Reviewer 3 (Remarks to the Author):

Thank you for asking! I checked the reply and feel that the authors have sufficiently addressed the reviewer's concerns.

Editorial Board Member checked revision on behalf of Reviewer 3 (Remarks to the Author):

Thank you for your patience. I have now carefully reviewed the authors' rebuttal letter and the revised manuscript in light of Reviewer #3's comments. In my assessment, the authors have satisfactorily addressed Reviewer #3's concerns. Importantly, they have substantially strengthened the manuscript by:

- Clearly defining and subtyping the plasmid as IncM2 (resolving the IncL/M ambiguity and aligning with the Carattoli framework).
- Performing long-read sequencing to structurally resolve the epidemic plasmid and characterize integron configurations.
- Conducting conjugation assays to demonstrate functional transferability of the IncM2 plasmid, thereby moving beyond purely descriptive genomic analysis.
- Clarifying the clonal relatedness between environmental and clinical isolates.
- Improving outbreak terminology and refining the definition of transmission clusters.
- Addressing the AST subset explanation and methodological clarifications raised in the general comments.
- The addition of long-read sequencing and conjugation experiments, in particular, directly addresses Reviewer #3's primary concern regarding the mechanistic investigation of the epidemic plasmid driving blaIMP-4 dissemination.

I do not see any remaining substantive scientific issues raised by Reviewer #3 that would preclude progression of the manuscript