Author’s response to reviews

Title: NDM-1 metallo-beta-lactamase and ArmA 16S rRNA methylase producing Providencia rettgeri clinical isolates in Nepal

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Author’s response to reviews: see over
Responses to editor's comment:

"Your manuscript has now been revised by four reviewers who found merit in your data. However, all reviewers have suggested various modifications, some of which are Major Compulsory Revisions. If you are willing to accept their suggestions, please revise your manuscript accordingly and send it back to us for analyses."

Response:
Thank you very much for your positive comments on our manuscript. We revised very carefully according to other reviewer’s comments.

Editorial requirements:

-- Please clarify if samples taken as part of standard patient care.

Response:
We added the sentence in the Methods section in lines 88-89 as follows; “Samples were obtained as part of standard patient care.”

-- Requesting deposition of data:

Nucleic acid sequences, protein sequences, and atomic coordinates should be deposited in an appropriate database in time for the accession number to be included in the published article. In computational studies where the sequence information is unacceptable for inclusion in databases because of lack of experimental validation, the sequences must be published as an additional file with the article. Where appropriate, authors should adhere to the standards proposed by the Microarray Gene Expression Data Society (http://www.mged.org) and must deposit microarray data in MIAME-compliant format in one of the public repositories, such as ArrayExpress (http://www.ebi.ac.uk/arrayexpress), Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/projects/geo/) or the Center for Information Biology Gene Expression Database (CIBEX; http://cibex.nig.ac.jp).

Response:
The nucleotide sequences surrounding bla_{NDM-1} was deposited in GenBank. We therefore added the sentence in lines 121-122 as follows; “The nucleotide sequences surrounding bla_{NDM-1} has been deposited in GenBank with the accession number AB828598.”
Responses to reviewers comments

Reviewer comments:

Reviewer #1 (Comments for the Author):
This is the first report of NDM-1, OXA-72 and armA from Providencia rettgeri from Nepal. This adds incremental knowledge to the worldwide geographical and species distribution of these resistance determinants.

Major Compulsory Revisions
Has OXA-72 been described in any species other than Acinetobacter before? If not, this should be highlighted and discussed.

Response:

OXA-72 producer were reported to be *Acinetobacter* spp. and *Klebsiella pneumonia*. We therefore added the sentences in the Discussion in lines 183-186 as follows;

“OXA-72 producers had been reported to be only *Acinetobacter* spp [27-37]. Recently, OXA-72 producing *K. pneumonia* isolates were deposited in GenBank in 2012 (Accession no. JX268653) as well as in 2013 (Accession no. AB825955).”

Did the authors obtain the entire sequences for the resistance genes sought? Did all these sequences match 100% with those of genes they say were found? Will the genome sequences be submitted to Genbank? If so, accession numbers should be given.

Response:

We obtained the entire sequences of the following genes as described in lines 107-112; “CLC genomics workbench version 5.5 (CLC bio, Tokyo, Japan) was used to perform de novo assembly of reads and search the 923 drug-resistance genes, including genes encoding β-lactamases, 16S rRNA methylases and aminoglycoside-acetyl/adenyltransferases, as well as point mutations in the *gyrA, parC* and *pmrCAB* operons, and in the *fos* genes, including *fosA, fosA2, fosA3, fosC* and *fosC2.*”

The sequences of drug-resistance genes tested matched 100%. We therefore added the sentence in lines 152-153 as follows; “All sequences of the drug-resistant genes tested were identical to those registered in GenBank.”

We added a sentence in lines 121-122 as follows; “The nucleotide sequences surrounding *blaNDM-1* has been deposited in GenBank with the accession number
AB828598."

Under the discussion, TEM-1 is not an extended-spectrum beta-lactamase and cannot explain the resistance to ceftazidime, cefepime and aztreonam. Another explanation must be found.

Response:
We re-analyzed the sequence data to explain the resistant to ceftazidime, cefepime and aztreonam, and then detected blaVEB-1 in all isolates tested. We revised the sentences in the Result section in lines 143-144 as follows; “All isolates had blaVEB-1, blaOXA-10, blaTEM-1, armA and aadA1; 3 had blaNDM-1; and 1 had blaOXA-72.”, and in lines 144-148 as follows; “None of these isolates had any other β-lactamase encoding genes, including the class A genes blaSHVs and blaCTX-Ms; the class B genes blaAIM, blaDIM, blaFIM, blaGIM, blaIMP, blaIND, blaKHM, blaSIM, blaSMB, blaSPM, blaTMBs, and blaVIM; the class C gene blaADCs (ampC); or the class D gene blaOXA except for blaOXA-10 and blaOXA-72.”, and in the Discussion section in lines 168-171 as follows; “The high MICs of all 5 isolates to ceftazidime, cefepime and aztreonam were likely due to the presence of blaVEB-1 [20], and the presence of armA in these isolates was likely associated with their extremely high resistance to all aminoglycosides tested [10].”

In the text, the PFGE patterns should be related to the antimicrobial resistance profile and resistance gene profile in the text rather than mentioning each item separately. For example is the isolate that did not have NDM-1 or OXA-72 the one with the PFGE profile that was slightly different? And is this the same isolate that had a lower MIC to the carbapenems?

Response:
NDM-1 and OXA-72 encoding genes in pathogenic bacteria are located within transferable plasmids. Therefore, the absence of methylase genes was found in the same PFGE cluster. We added the sentences in the lines 200-202 as follows; “Since blaNDM-1 and blaOXA-72 in P. rettgeri are located in transferable plasmids, the absence of carbapenemase encoding genes was found in the same PFGE clusters.”

There is a problem viewing the far left of the table in the version of the manuscript I downloaded.

Response:
We are sorry. The table was edited to avoid the view problem.
Minor Essential Revisions
There is a repetition in the Background "P. rettgeri has been associated with nosocomial infections..."
Response:
We revised the sentence in the Background in the Abstract in lines 39-41 as follows; “Drug-resistant Providencia rettgeri producing metallo-β-lactamase and 16S rRNA methylase has been reported in several countries. We analyzed P. rettgeri clinical isolates with resistance to carbapenems and aminoglycosides in a hospital in Nepal.”

Discretionary Revisions
NDM-1 has been described in P. rettgeri before so for the amount of new information presented, I feel the manuscript could be rewritten with the brevity of new data correspondence.
Response:
We revised and shortened the manuscript. We revised the redundant sentences which other reviewers pointed out, and the Result section of antibiotic susceptibility in a concise way.

The manuscript should be more focused for example it is not worth mentioning the rare cases of P. rettgeri infection in defibrillators and xanthogranulomatous pyelonephritis. In the discussion why mention the pmrCAB and fos gene mutations when none were found in the isolates.
Response:
We deleted the sentence on case reports from the Background section, and deleted the sentence mentioning the pmrCAB and fos gene mutation from the Discussion section.

Under drug-resistant genes. Were the genes sought by specific PCR or by whole genome sequencing. If by whole genome sequencing, it is not meaningful to list some of the beta-lactamase genes that were not found. It is just necessary to mention what was found.
Response:
Whole genome sequencing were performed by the next generation sequence (MiSeq) and analyzed by CLC genomics workbench version 5.5. We revised the sentence in lines 107-112 as follows; “CLC genomics workbench version 5.5 (CLC bio, Tokyo, Japan) was used to perform de novo assembly of reads and search the 923 drug-resistance genes, including genes encoding β-lactamases, 16S rRNA methylases and
aminoglycoside-acetyl/adenyltransferases, as well as point mutations in the \textit{gyrA}, \textit{parC} and \textit{pmrCAB} operons, and in the \textit{fos} genes, including \textit{fosA}, \textit{fosA2}, \textit{fosA3}, \textit{fosC} and \textit{fosC2}.

The antimicrobial susceptibilities are in the table so there is little need to repeat them in the text under results. Only results the authors particularly wish to highlight should be mentioned in the text.

Response:

We revised the sentences in lines 132-139 as follows; “Four of the 5 isolates were resistant to carbapenems (imipenem, doripenem and meropenem) and piperacillin/tazobactam and, whereas the remaining isolate was susceptible to piperacillin/tazobactam, doripenem and meropenem. The remaining one showed intermediate resistance to imipenem (Table 1). All 5 isolates were highly resistant to cephalosporins (ceftazidime and cefepime). All 5 isolates were highly resistant to aztreonam, aminoglycosides (arbekacin, amikacin and gentamicin), ciprofloxacin, colistin and fosfomycin. All five isolates showed intermediate resistance to tigecycline.”

Reviewer #2 (Comments for the Author):

This paper presents the isolation and characterization of five \textit{P. rettgeri} isolates with multiple resistance to antimicrobial agents. The authors describe, for the first time in Nepal, the co-production of NDM-1 or OXA-72 carbapenemases and ArmA methylase in the isolates. PFGE revealed that isolates were closely related.

The paper presents interesting data, but I believe that some points should be clarified before publication. I do recommend for the authors to consider the reviewer suggestions and to submit a revised version of the manuscript.

**Major Compulsory Revisions**

- Introduction is not enough concise and must be reviewed. Authors repeated several times that “\textit{P. rettgeri} has been isolated…” (page 5, line 62), “\textit{P. rettgeri} has been associated…” (p. 5, l. 63), “\textit{P. rettgeri}” has been associated…” (p. 5, line 65).

Response:

We revised the sentences in lines 64-67 as follows; “\textit{Providencia rettgeri} has been associated with nosocomial infections, including catheter-related urinary infections, bacteremia, skin infections, diarrhea, and gastroenteritis [1, 2]. \textit{P. rettgeri} acquired drug
resistance represents a challenge to therapeutics.”

- The sentence “P. rettgeri has been associated with nosocomial infections (…) diarrhea, and gastroenteritis” (line 65-67) is duplicated and must be deleted.

  Response:  
  We are sorry for the redundant sentence. We deleted that.

- The authors should emphasize the relates of P. rettgeri in human infections, as they describe in the lines 63-65 and 67-70 (page 5) instead of valorizing the animal and environmental sources.

  Response:  
  We deleted the description about P. rettgeri from animal and environmental sources to emphasize the relation of P. rettgeri in human infections.

- I suggest to include a link sentence between “… Korea.” and “To date, …” (p. 5, l. 70). Something like this: “Bacterial drug resistance represents a challenge to therapeutics”.

  Response:  
  We added the sentence in lines 66-67 as follows; “P. rettgeri acquired drug resistance represents a challenge to therapeutics.”

- The sentence “We describe here. … in Nepal” (p. 5, l. 73-75) should be moved to the end of introduction.

  Response:  
  We moved the sentence to the end of introduction in lines 81-82.

- Page 5, line 77. It is known that NDM emergence started in New Dehli, India (Antimicrob Agents Chemother 2009; 53: 5046–54), and, despite the patient be Swedish, he had traveled to that city. The authors should change that the NDM was initially identified in Sweden, and include the reference above mentioned.

  Response:  
  We changed the reference.

- Page 6, lines 87-89: authors have written that ArmA is widely distributed, and, due to this reason, they do not need to list the countries; just place the reference at the end of the sentence.

  Response:
We revised the sentences in lines 76-78 as follows; “Gram-negative pathogens producing 16S rRNA methylase ArmA have been isolated in various countries [10].”


Response:
We added the sentence in lines 79-80 as follows; “Co-production of several resistance determinants is not rare in Enterobacteriaceae [11-15], but in P. rettgeri it is a phenomenon seldom observed [5].”

- In the end of introduction, place the sentence “We describe... Nepal” (p. 5, l. 73-75).

Response:
We moved the sentence to the end of introduction in lines 81-82.

- Page 6, lines 95-96: references for phenotypical and genetic identification must be included.

Response:
We added the references.

- Page 7, line 107: MIC values were categorized in susceptible, intermediate or resistance, but this information is not in this section. Authors must state which breakpoints they used (CLSI or EUCAST, for example). A reference (if CLSI) must me added (maybe M100-S22 or S23).

Response:
We added the sentence in the Methods section in lines 101-102 as follows; “Breakpoints to antibiotics were determined by the CLSI guidelines (M100-S22).”

- Page 7, line 112: authors should describe how they performed this analysis. Did they employ some software?

Response:
We revised the sentences in lines 107-112 as follows; “CLC genomics workbench
version 5.5 (CLC bio, Tokyo, Japan) was used to perform de novo assembly of reads and search the 923 drug-resistance genes, including genes encoding β-lactamases, 16S rRNA methylases and aminoglycoside-acetyl/adenyltransferases, as well as point mutations in the gyrA, parC and pmrCAB operons, and in the fos genes, including fosA, fosA2, fosA3, fosC and fosC2.”

- Pages 8-9, lines 130-141: if the information regarding antimicrobial susceptibility is already in the table (I could not see the entire table, it is cut), it should not be repeated in the text. On the other hand, describe the resistance patterns should add information to the manuscript.

Response:
We revised the sentences in lines 132-139 as follows; “Four of the 5 isolates were resistant to carbapenems (imipenem, doripenem and meropenem) and piperacillin/tazobactam and, whereas the remaining isolate was susceptible to piperacillin/tazobactam, doripenem and meropenem. The remaining one showed intermediate resistance to imipenem (Table 1). All 5 isolates were highly resistant to cephalosporins (ceftazidime and cecepmime). All 5 isolates were highly resistant to aztreonam, aminoglycosides (arbekacin, amikacin and gentamicin), ciprofloxacin, colistin and fosfomycin. All five isolates showed intermediate resistance to tigecycline.”

- Page 7, line 150: clarify that other blaOXA variants than blaOXA-72 were not identified.

Response:
We revised the sentence in lines 147-148 as follows; “…the class D gene blaOXAs except for blaOXA-10 and blaOXA-72.”

- Page 10, line 162: Discussion is very concise but it does not include the results of structure surrounding blaNDM gene (p. 10, l. 156-160). What are the implications of this finding?

Response:
We added the paragraph in the Discussion in lines 190-197 as follows; “The blaNDM-1 in the P. rettgeri isolates in Nepal will come from Enterobacteriaceae or glucose-nonfermentative Gram-negative spp., because the genetic environment surrounding blaNDM-1 has been reported in other Gram-negative spp., including Citrobacter freundii plasmid pYE315203 detected in China (Accession no. JX254913), E. coli plasmid pNDM102337 in Canada (Accession no. JF714412), K. pneumoniae
plasmids pKPX-1, pKPN5047 and pNDM-HN380 in China (Accession nos. AP012055, KC311431 and JX104760, respectively), and \textit{P. rettgeri} plasmid pFR90 (Accession no. JQ362415) in China.”

- Page 13. Table is not complete in my pdf version. Please verify.
Response:
We are sorry. The table was edited to avoid the view problem.

**Minor Essential Revisions**
- Page 5, line 7. Enterobacteriaceae is a family name, and, due to this reason, must not be italicizing. Same in page 6, line 87.
Response:
We are very sorry. We revised the typos.

- Page 6, line 79. The nomenclature for \textit{bla} genes should be written with the allele subscript. This can be easily done by selecting the text and pressing the keys [Ctrl] plus [+] in MS Word. The same observation for other alleles in the text.
Response:
We are very sorry. We revised the typos.

- Page 6, lines 82-84. I do not believe that is important to describe all the countries that have already isolated OXA-72-producing strains. Replace the list of the countries for “worldwide” and keep the references at the end.
Response:
We revised the sentence as follows; “Since then, \textit{Acinetobacter} spp. harboring \textit{bla}_{OXA-72} have been reported in various countries [27-37].”, and moved this sentence from the Background to Discussion section in lines 188-189.

- Page 6, line 95: replace “were sputum” for “from sputum”.
Response:
We replace “were sputum” for “from sputum” in line 88.

- Page 6, lines 95-96: I suggest to change the text for: “Phenotypical identification (REFERENCE) was confirmed by partial (?) 16S rRNA DNA sequencing (REFERENCE)”.
Response:
We revised the sentence in lines 89-90 as follows; “Phenotypical identification [16] was confirmed by partial 16S rRNA sequencing [17, 18].”

- Page 8, line 119: Replace “Unweighted Pair Group Method” for “Unweighted Pair Group Method with Arithmetic Mean” if the authors analyzed by UPGMA.
  
  **Response:**
  
  We revised the sentence in line 115-118 as follows; “PFGE analysis was performed as described [3]. Fingerprinting patterns were analyzed by the unweighted Pair Group Method with Arithmetic Mean using Molecular Analyst Fingerprinting Plus software (Bio-Rad Laboratories, Hercules, CA, USA) to create an average linkage-based dendrogram.”

**Discretionary Revisions**

- Page 11, line 180: resistance mechanisms to polymyxin B and fosfomycin are well elucidated so far?
  
  **Response:**
  
  Resistance mechanisms to polymyxin B are not well elucidated, whereas fosfomycin modification enzymes fosA and fosC were associated with fosfomycin resistance.

**Reviewer #3 (Comments for the Author):**

1. Is the question posed by the authors well defined? Partially

2. Are the methods appropriate and well described? Partially

3. Are the data sound? Yes

4. Does the manuscript adhere to the relevant standards for reporting and data deposition? Yes

5. Are the discussion and conclusions well balanced and adequately supported by the data? Yes, but must be improved.

6. Are limitations of the work clearly stated? No.

7. Do the authors clearly acknowledge any work upon which they are building, both
This is a descriptive study reporting the presence of NDM-1 gene as well as OXA-72 in P. rettgeri isolates recovered in Nepal.

It is an interesting and relevant study for two reasons: the description of NDM-1 in Nepal from an epidemiological point of view, and the presence of OXA-72 in an Enterobacteriaceae isolate that to the best of my knowledge is the first description in an organism other than an Acinetobacter spp. Both are very important findings presented by the authors. However, none of these topics were properly explored by them in the discussion.

Response:
Thank you for your comments on our manuscript. We revised the manuscript including the Discussion section.

Major compulsory revision:
1. Re-write the abstract. The background presented does not justify the study. Results: 5 were carbapenem-resistant isolates, in 4 an enzyme was detected, what about the other? It is absolutely not clear (as in the results section in the text) if the 5th isolate which was not identical to the other four was that which had no enzyme detected, was a NDM one or the OXA-72. Conclusions: The detection of OXA-72 in a P. rettgeri must be highlighted.

Response:
We revised the abstract as follows;

Background
Drug-resistant Providencia rettgeri producing metallo-β-lactamase and 16S rRNA methylase has been reported in several countries. We analyzed P. rettgeri clinical isolates with resistance to carbapenems and aminoglycosides in a hospital in Nepal.

Methods
Five clinical isolates of multidrug-resistant P. rettgeri were obtained in a hospital in Nepal. Antimicrobial susceptibilities were determined using the microdilution method
and entire genomes were sequenced to determine drug-resistant genes. Epidemiological analysis was performed by pulsed-field gel electrophoresis. Pulsed-field gel electrophoresis pattern was analyzed by the unweighted-pair-group method to create an average linkage-based dendrogram.

**Results**

Four of the 5 isolates were resistant to carbapenems (imipenem and meropenem), with MICs $\geq 16$ mg/L, with the remaining isolate showing intermediate resistance to imipenem, with an MIC of 2 mg/L and susceptibility to meropenem with an MIC $\leq 1$ mg/L. All 5 isolates had $bla_{VEB-1}$. Of the 4 carbapenem resistant strains, 3 had $bla_{NDM-1}$ and 1 had $bla_{OXA-72}$. All isolates were highly resistant to aminoglycosides (MICs $> 1,024$ mg/L) and harbored armA. As the result of pulsed-field gel electrophoresis pattern analysis in the 5 $P. rettgeri$ isolates, 4 had identical PFGE patterns and the fifth showed 95.7% similarity.

**Conclusions**

This is the first report describing multidrug-resistant $P. rettgeri$ strains harboring $bla_{NDM-1}$ or $bla_{OXA-72}$ and armA isolated from patients in Nepal.

2. **Background:**

   a. All this section should be re-written in a logical manner, highlighting the epidemiological importance of NDM and what was the objective of the authors before the positive results for these enzymes (including OXA-72).

   **Response:**

   We revised this section as follows:

   *Providencia rettgeri* has been associated with nosocomial infections, including catheter-related urinary infections, bacteremia, skin infections, diarrhea, and gastroenteritis [1, 2]. *P. rettgeri* acquired drug resistance represents a challenge to therapeutics. To date, there have been 4 reports of *P. rettgeri* isolates harboring metallo-β-lactamase (MBL) encoding genes, including IMP-type MBL producers in Japan [3, 4]; VIM-type MBL, PER-1 extended-spectrum β-lactamase (ESBL) and 16S rRNA methylase ArmA in Korea [5]; and NDM-type MBL in Israel [6].

   NDM-type MBL was initially identified in *Klebsiella pneumoniae* and *Escherichia coli* in 2009 in Sweden [7]. Since then, NDM-1-producing Enterobacteriaceae have been isolated in various parts of the world [8, 9].

   Exogenously acquired 16S rRNA methylase genes responsible for very high levels of resistance to various aminoglycosides are widely distributed among Enterobacteriaceae and glucose-nonfermentative microbes [10]. Gram-negative
pathogens producing 16S rRNA methylase ArmA have been isolated in various countries [10].

Co-production of several resistance determinants is not rare in Enterobacteriaceae [11-15], but in P. rettgeri it is a phenomenon seldom observed [5]. We describe here P. rettgeri clinical isolates producing carbapenemase (NDM-1 or OXA-72) and 16S rRNA methylase (ArmA) in Nepal.

b. The last two paragraphs of the background section must be replaced. They should be placed before the sentence “We describe here 3 P. rettgeri isolates producing NDM-1 and ArmA and 1 isolate producing OXA-72 and ArmA from a hospital in Nepal.”
Response:
We revised these paragraphs as follows;

Exogenously acquired 16S rRNA methylase genes responsible for very high levels of resistance to various aminoglycosides are widely distributed among Enterobacteriaceae and glucose-nonfermentative microbes [10]. Gram-negative pathogens producing 16S rRNA methylase ArmA have been isolated in various countries [10].

Co-production of several resistance determinants is not rare in Enterobacteriaceae [11-15], but in P. rettgeri it is a phenomenon seldom observed [5]. We describe here P. rettgeri clinical isolates producing carbapenemase (NDM-1 or OXA-72) and 16S rRNA methylase (ArmA) in Nepal.

3. Methods:
a. The authors state that “Genomic sequences were examined to detect genes encoding #-lactamases, 16S rRNA methylases and aminoglycoside-acetyl/adenyltransferases, as well as point mutations in the gyrA, parC and pmrCAB operons, and in the fos genes…” but no mention is done for the beta-lactamase genes. In the results, the authors mention a lot of bla genes, but there is no reference to the method used for detection. I suppose it was a PCR (or analysis of the genome sequencing). It must be shown, and if PCR, which primer was used to detect the enzymes, especially NDM-1 and OXA-72.
Response:
We are sorry for these confusing sentences. In the present study, we performed the whole genome sequencing by the next generation sequencer, MiSeq. We therefore revised the sentences in the Materials and Methods in lines 107-112 as follows; “CLC genomics workbench version 5.5 (CLC bio, Tokyo, Japan) was used to perform de novo assembly of reads and search the 923 drug-resistance genes, including genes encoding
β-lactamases, 16S rRNA methylases and aminoglycoside-acetyl/adenyltransferases, as well as point mutations in the gyrA, parC and pmrCAB operons, and in the fos genes, including fosA, fosA2, fosA3, fosC and fosC2.”

4. Discussion: The discussion is poor. There are just comments on obvious associations between phenotypic and genotypic findings. The importance of the paper relies on the epidemiological finding and the detection of OXA-72 in an Enterobacteriaceae. It must be more deeply discussed in this section.

Response: We revised the paragraph in the Discussion section in lines 179-189 as follows; “NDM-1 and/or OXA-72 will be associated with carbapenem-resistance in P. rettgeri in Nepal. This is the first report of NDM-1 producing P. rettgeri clinical isolates in Nepal. Gefen-Halevi et al [6] recently reported that P. rettgeri clinical isolates harboring blaNDM-1 were obtained from Israeli patients. This is also the first report of OXA-72 producing P. rettgeri clinical isolates. OXA-72 producers had been reported to be only Acinetobacter spp [27-37]. Recently, OXA-72 producing K. pneumonia isolates were deposited in GenBank in 2012 (Accession no. JX268653) as well as in 2013 (Accession no. AB825955). OXA-72 is a class D carbapenemase; its gene, blaOXA-72, was first identified in an Acinetobacter baumannii strain isolated in 2004 in Thailand (Accession no. AY739646). Since then, Acinetobacter spp. harboring blaOXA-72 have been reported in various countries [27-37].”

We added the paragraph on the genetic environment of blaNDM-1 in the Discussion section in lines 190-197 as follows; “The blaNDM-1 in the P. rettgeri isolates in Nepal will come from Enterobacteriaceae or glucose-nonfermentative Gram-negative spp., because the genetic environment surrounding blaNDM-1 has been reported in other Gram-negative spp., including Citrobacter freundii plasmid pYE315203 detected in China (Accession no. JX254913), E. coli plasmid pNDM102337 in Canada (Accession no. JF714412), K. pneumoniae plasmids pKPX-1, pKPN5047 and pNDM-HN380 in China (Accession nos. AP012055, KC311431 and JX104760, respectively), and P. rettgeri plasmid pFR90 (Accession no. JQ362415) in China.”

Minor Essential revision
Background:
a. Third sentence is a repetition of the second one.
Response: We deleted the redundant sentence.
b. Why the following sentence? “There were case reports of P. rettgeri infections, such as an automatic implantable cardioverter defibrillator infection in the United States of America [7] and a case of xanthogranulomatous pyelonephritis in Korea [8].” There are many cases of P. rettgeri infections; there is no sense in referring to case reports.

Response:
The sentence was deleted.

c. “To date, there have been 4 reports of P. rettgeri isolates carrying drug-resistant genes, including IMP-type metallo-β-lactamase (MBL) producers in Japan [9, 10]; VIM-type MBL, PER-1 extended-spectrum β-lactamase (ESBL) and 16S rRNA methylase ArmA in Korea [11]; and NDM-type MBL in Israel [12].” Actually, there are other reports of P. rettgeri carrying “drug-resistant genes”. I suppose the authors are referring to carbapenemase-encoding genes.

Response:
We were mainly referring to metallo-β-lactamase producing P. rettgeri in the sentence. We therefore revised the sentence in lines 67-70 as follows; “To date, there have been 4 reports of P. rettgeri isolates harboring metallo-β-lactamase (MBL) encoding genes, including IMP-type MBL producers in Japan [3, 4]; VIM-type MBL, PER-1 extended-spectrum β-lactamase (ESBL) and 16S rRNA methylase ArmA in Korea [5]; and NDM-type MBL in Israel [6].”

Methods:
a. What is exactly the “pus samples”? From which site they were obtained?

Response:
We revised the sentence in lines 86-89 as follows; “Five P. rettgeri clinical isolates were obtained from 5 patients in May to July 2012 at Tribhuvan University Teaching Hospital in Kathmandu, Nepal, including 3 isolates from sputum and 2 from pus at surgical wound sites. Samples were obtained as part of standard patient care.”

b. Although the isolates were identified by sequencing, it would be appropriate how was the “phenotypic” method used for identification (line 95)

Response:
We revised the sentence in lines 89-90 as follows; “Phenotypical identification [16] was confirmed by partial 16S rRNA sequencing [17, 18].”
Results:
a. The results section starts with the description of the susceptibility profile of the isolates to piperacillin-tazobactam. Re-write the paragraph replacing the results of carbapenem MICs first.

Response:
We revised the paragraph in lines 132-139 as follows; “Four of the 5 isolates were resistant to carbapenems (imipenem, doripenem and meropenem) and piperacillin/tazobactam and, whereas the remaining isolate was susceptible to piperacillin/tazobactam, doripenem and meropenem. The remaining one showed intermediate resistance to imipenem (Table 1). All 5 isolates were highly resistant to cephalosporins (ceftazidime and cefepime). All 5 isolates were highly resistant to aztreonam, aminoglycosides (arbekacin, amikacin and gentamicin), ciprofloxacin, colistin and fosfomycin. All five isolates showed intermediate resistance to tigecycline.”

Reviewer #4 (Comments for the Author):
Manuscript title: NDM-1 metallo-β-lactamase and ArmA 16S rRNA methylase producing Providencia rettgeri clinical isolates in Nepal. Corresponding author: Dr. Teruo Kirikae.
The authors reported the characterization of 5 P. rettgeri isolated from patient samples in Nepal.
While the topic of the paper is interesting topic, the reviewer has major concerns with the manuscript as presented.

Major Compulsory Revisions:
1. The paper needs major English language editing. There were repetitions of sentences as in lines 63-65 and lines 65-57. Some of the information presented was very confusing such as on lines 43, 45, 53-54, 95.
Response:
The manuscript was edited by native English. The redundant sentences in lines 65-67 in original manuscript were revised. The confusing sentences were deleted.

2. The paper as presented is long and can be shortened. The reviewer recommends that
the authors restructure the paper to a short report or letter to the editor.

Response:
The manuscript was shortened in a concise way.

3. The authors need to clarify how many isolates they are trying to characterize. On line 43, the authors indicated that they obtained 5 isolates, however, one lines 73-75, the authors mention that they are describing a total of 4 isolates (We describe here 3 P. rettgeri isolates producing NDM-1 and ArmA and 1 isolate producing OXA-72 and ArmA from a hospital in Nepal. Please clarify.

Response:
A total of 5 Providencia rettgeri isolates were obtained in a hospital in Nepal. Of them, 4 isolates were highly resistant to carbapenems and 1 isolate was intermediate to carbapenems. Of 4 isolates with highly resistance to carbapenems, 3 had \textit{bla}_{NDM-1} and 1 had \textit{bla}_{OXA-72}. We revised the manuscript in lines 81-82 as follows; “We describe here \textit{P. rettgeri} clinical isolates producing carbapenemase (NDM-1 or OXA-72) and 16S rRNA methylase (ArmA) in Nepal.”

4. Lines 42-46. In the methods section of the abstract, please better clarify the methodologies used in the study. Did the authors use next generation sequencing? If so, which platform? Please add that the CLSI guidelines were followed. Why the authors did not report the ertapenem results of these isolates? Isn’t ertapenem recommended by the CLSI to detect carbapenemase producing bacteria?

Response:
The platform of next generation sequencing was MiSeq system (Illumina, San Diego, CA), and de novo assemble and detection of antibiotic resistance genes were performed by using CLC genomics workbench version 5.5 (CLC bio, Tokyo, Japan). We revised the sentence in lines 107-112 as follows; “CLC genomics workbench version 5.5 (CLC bio, Tokyo, Japan) was used to perform de novo assembly of reads and search the 923 drug-resistance genes, including genes encoding \(\beta\)-lactamases, 16S rRNA methylases and aminoglycoside-acetyl/adenyltransferases, as well as point mutations in the \textit{gyrA}, \textit{parC} and \textit{pmrCAB} operons, and in the \textit{fos} genes, including \textit{fosA}, \textit{fosA2}, \textit{fosA3}, \textit{fosC} and \textit{fosC2}.”

On antibiotics susceptibility test, the information of CLSI guideline was added in the revised manuscript. We added the sentence in lines 101-102 as follows; “Breakpoints to antibiotics were determined by the CLSI guidelines (M100-S22).”
Ertapenem is not available in Japan. Alternatively, we performed the drug susceptibility test for doripenem. We revised the sentence in the Materials and Methods section in lines 99-100 as follows; “…and doripenem (Shionogi, Osaka, Japan) were determined using the microdilution method,…”, and in the Result section in lines 132-139 as follows; “Four of the 5 isolates were resistant to carbapenems (imipenem, doripenem and meropenem) and piperacillin/tazobactam and, whereas the remaining isolate was susceptible to piperacillin/tazobactam, doripenem and meropenem. The remaining one showed intermediate resistance to imipenem (Table 1). All 5 isolates were highly resistant to cephalosporins (ceftazidime and cefepime). All 5 isolates were highly resistant to aztreonam, aminoglycosides (arbekacin, amikacin and gentamicin), ciprofloxacin, colistin and fosfomycin. All five isolates showed intermediate resistance to tigecycline.”, and in the Discussion section in lines 164-166 as follows; “The relatively high MICs of these isolates, except for IONTU94, to piperacillin/tazobactam, imipenem, doripenem and meropenem were likely due to the presence of blaNDM-1 or blaOXA-72 (Table 1).”

5. Table 1 is poorly presented. The reviewer could not see the whole table. The isolates name where not indicated on the table.

Response:
We are sorry. The table was edited to avoid the view problem.

6. Line 168. In the discussion, the authors need to review the activity of blaTEM-1. blaTEM-1 is a beta-lactamase similar to blaTEM-2. It is not an extended spectrum beta lactamases. Thus blaTEM-1 can not be the enzyme responsible for inactivating ceftazidime and cefepime, ext.…

Response:
We re-analyzed the sequence data to explain the resistant to ceftazidime, cefepime and aztreonam, and then detected blaVEB-1 in all isolates tested. We revised the sentences in the Result section in lines 143-144 as follows; “All isolates had blaVEB-1, blaOXA-10, blaTEM-1, armA and aadA1; 3 had blaNDM-1; and 1 had blaOXA-72.”, and in lines 144-148 as follows; “None of these isolates had any other ß-lactamase encoding genes, including the class A genes blaSHVs and blaCTX-Ms; the class B genes blaAMs, blaDIM, blaFIM, blaGIM, blaIMP, blaINDs, blaKHM, blaSIM, blaSMB, blaTMBs and blaVIMs; the class C gene blaADCs (ampC); or the class D gene blaOXAAs except for blaOXA-10 and blaOXA-72.”, and in the Discussion section in lines 168-171 as follows; “The high MICs of all 5 isolates to ceftazidime, cefepime and aztreonam were likely due to the presence of blaVEB-1 [20],
and the presence of armA in these isolates was likely associated with their extremely high resistance to all aminoglycosides tested [10].”

7. In the discussion section, the authors need to discuss the data in the context of what was reported in other parts of the World. The authors need to address if these isolates were a result of hospital acquired infections.

Response:
We added the paragraph in the Discussion section in lines 179-189 as follows; “NDM-1 and/or OXA-72 will be associated with carbapenem-resistance in P. rettgeri in Nepal. This is the first report of NDM-1 producing P. rettgeri clinical isolates in Nepal. Gefen-Halevi et al [6] recently reported that P. rettgeri clinical isolates harboring blaNDM-1 were obtained from Israeli patients. This is also the first report of OXA-72 producing P. rettgeri clinical isolates. OXA-72 producers had been reported to be only Acinetobacter spp [27-37]. Recently, OXA-72 producing K. pneumonia isolates were deposited in GenBank in 2012 (Accession no. JX268653) as well as in 2013 (Accession no. AB825955). OXA-72 is a class D carbapenemase; its gene, blaOXA-72, was first identified in an Acinetobacter baumannii strain isolated in 2004 in Thailand (Accession no. AY739646). Since then, Acinetobacter spp. harboring blaOXA-72 have been reported in various countries [27-37].”

Minor Essential Revisions
1. Please move lines 73-75 to the end of the background section and elaborate a bit about what the goal of the paper is.

Response:
We moved the sentence to the end of the background section and revised the sentence in lines 79-82 as follows; “Co-production of several resistance determinants is not rare in Enterobacteriaceae [11-15], but in P. rettgeri it is a phenomenon seldom observed [5]. We describe here P. rettgeri clinical isolates producing carbapenemase (NDM-1 or OXA-72) and 16S rRNA methylase (ArmA) in Nepal.”

2. Line 45-46 of the abstract methods section, please clarify. Epidemiological analysis is a very general term. Please be specific when talking about PFGE.

Response:
We revised the sentence in lines 45-48 as follows; “Epidemiological analysis was performed by pulsed-field gel electrophoresis. Pulsed-field gel electrophoresis pattern
was analyzed by the unweighted-pair-group method to create an average linkage-based dendrogram.”

3. Lines 95-96 of the bacterial strains sections of the methods, please clarify which identification system was used to phenotypically used to identify the bacteria.
   Response:
   We revised the sentence and added the references in lines 89-90 as follows;
   “Phenotypical identification [16] was confirmed by partial 16S rRNA sequencing [17, 18].”

4. Lines 156-160 of the Genomic structure section. Please clarify what was actually done. Please add a reference for the methodology used.
   Response:
   We revised the Materials and Methods section in lines 107-112 as follows; “CLC genomics workbench version 5.5 (CLC bio, Tokyo, Japan) was used to perform de novo assembly of reads and search the 923 drug-resistance genes, including genes encoding β-lactamases, 16S rRNA methylases and aminoglycoside-acetyl/adenyltransferases, as well as point mutations in the gyrA, parC and pmrCAB operons, and in the fos genes, including fosA, fosA2, fosA3, fosC and fosC2.”

Reviewer #5 (Comments for the Author):
This is a straightforward, well written short report describing the dissemination of the NDM-1 metallo-beta-lactamase amongst Providencia rettgeri in Nepal. The authors have used whole genomic sequencing to rapidly define the resistome of the isolates, demonstrating the clinical utility of this approach. The study also identifies the likely genetic basis of intrinsic polymyxin and fosfomycin resistance in this species. The experimental methods are adequately described and the results and their interpretations clearly presented. The article appears suitable for publication in its current format.
Response:
Thank you very much for your positive comments on our manuscript. We revised very carefully according to other reviewer’s comments.