Author's response to reviews

Title: Enhanced surveillance of invasive listeriosis in the Lombardy region, Italy, in the years 2006-2010 reveals major clones and an increase in serotype 1/2a

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Author's response to reviews: see over
Dear Editor,

I am submitting the revised version of the manuscript by Mammina C et al. entitled “A snapshot from ongoing enhanced surveillance of invasive listeriosis in Lombardy, Italy, 2006-2010”. Please, note that the title has been changed to: “Enhanced surveillance of invasive listeriosis in the Lombardy region, Italy, in the years 2006-2010 reveals major clones and an increase in serotype 1/2a”.

I am attaching below the point-by-point answers to the comments of reviewers.

I hope the manuscript in the present version could be considered for publication on BMC Infectious Diseases.

Sincerely

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Answers to reviewers

Reviewer 1: Stephen Knabel

Reviewer's report:

Major Compulsory Revisions:
1. The title needs to be more specific to indicate the conclusions reached, not just the type of study conducted. For example, the title might better read “Enhanced surveillance of invasive listeriosis in the Lombardy region of Italy from 2006-2010 reveals major clones and an increase in serotype 1/2a”

The suggestion of the reviewer has been accepted and the title has been changed accordingly.

2. “Enhanced surveillance” needs to be more clearly defined in the introduction. Does this include conventional epidemiology or just Laboratory-based molecular epidemiology? Throughout the manuscript the authors state that no listeriosis outbreaks were notified in the Lombardy Region during the period in question (2006-2010), and they repeatedly emphasize the potential for “hidden” outbreaks to go undetected. The authors mention listeriosis outbreaks were detected in Europe in the past, were they ever detected in the Lombardy Region of Italy? If so, when and what was/were the food vehicles responsible? More discussion is needed about how conventional epidemiology was conducted in the Lombardy Region during the period in question. For example, in the U.S. epidemiologists often use shopper cards, which allows CDC to electronically track specific food items and brands purchased by ill and control customers, which allows them to perform more accurate case-control studies to identify the specific food vehicle responsible for a listeriosis outbreak. For example, the US CDC successfully detected the recent 2012 Italian Ricotta Cheese outbreak using a combination of conventional and molecular epidemiology – this two-pronged approach needs more emphasis in the discussion in this manuscript. Was this type of conventional epidemiology investigation being done routinely in the Lombardy Region of Italy during the period in question? If not, why not? Were case-control studies routinely being performed when these time and geographically-linked clusters were repeatedly detected by molecular epidemiology? Again, if not, why not. It is painfully obvious in this study that without good conventional epidemiology it is impossible to know the epidemiologic relevance of data from different subtyping methods. Therefore, specific recommendations concerning “enhanced” conventional epidemiology strategies need to be also included in the conclusions.

In Italy, the routine surveillance of infectious diseases implies generally only the mandatory notification to the health authority. In the event of a foodborne outbreak, the local department of prevention starts a conventional epidemiological investigation by performing descriptive and analytical studies and the environmental investigation plus food sampling. The routine investigation method, as we know, can be quite insensitive when applied to listeriosis. The surveillance system does work in a similar way in Lombardy too, where, however, it is likely working better than in other regions, as the notification rate suggests.

However, to identify an outbreak of listeriosis is not so obvious. Only, in the last years in some cases molecular epidemiology has being used to support conventional epidemiology, but this is not until today the “routine” investigation procedure.

What we define as “enhanced surveillance” is described in Methods. On the other hand, our study is a retrospective epidemiological study, not a report of the listeriosis surveillance by the regional health authorities. This is reason why we repeatedly say that no outbreaks have been reported and, consequently, some outbreaks have been very likely missed. We are aware, of course, of the
serious limitations of the conventional epidemiological approach in the “Italian” version and agree at all about the concerns expressed by the reviewer. Italy is far from US, but not only about the investigation of foodborne listeriosis. It is, indeed, to be taken into due consideration that the food chain (especially for some traditional products) from the production to the processing, marketing and distribution is different than that of US and it would be very impractical or inappropriate to investigate our setting by the methods (e.g. shopper cards) described by the reviewer. It is necessary, of course, to develop more effective epidemic intelligence services and methodologies.

3. Pulse-field gel electrophoresis of Lm isolates was conducted using AscI and ApaI; however, in the legend to Fig. 2 it says XbaI was used. This needs to be corrected.

This has been done.

4. Exactly how were the PFGE clusters defined? This needs to be inserted at the end of the PFGE section in the Materials and Methods on page 7.

This has been done.

5. The authors need to review ALL STs to make sure they were assigned to the correct clonal complexes. For example, the authors claim ST38 is in clonal complex 38, when in fact ST38 is in clonal complex 101 (see Ragon et al., 2008 and Chenal Francisque, 2011). In Fig. 2 one ECII isolate (code 233) has the same ST/CC (2) as ECIV isolates, but should be in CC6 (See Ragon et al., 2008). This is probably due to either a MLST or MVLST sequencing error or an error in assigning this isolate to CC2. This same concern occurs with ECIII isolate code 118 being assigned to CC14, which should be in CC11, like isolate code 226. Also, isolate code 191 is not listed as ECIII, but it has the same ST/CC (11) as ECIII (Ragon et al., 2008). The authors need to recheck their results and/or rerun their experiments to make sure that data in all Tables and Figures agree with one another and with the literature. If they disagree with the literature after rerunning MLST and/or MVLST, the possible reasons for these discrepancies need to be discussed in the Discussion section.

All the discrepant and inconsistent results have been checked and the manuscript, figures and tables modified accordingly.

6. The authors report finding ST8 and ST120 in CC8, which are most likely ECV. ECV-specific primers and the MVLST sequences for ECV have been published (Knabel et al., 2012; Gilmour et al., 2010). Therefore, these analyses need to be performed and this data entered into Table 2. If all 11 ST8 isolates listed in Fig. 3 are ECV, then this would increase the number of epidemic clones detected (from 3 to 4) and the total percentage of isolates that are epidemic clones (from 32% to 40%). This information needs to be included in the Materials and Methods, Results and Discussion.

This has been done.

7. An outbreak due to ECIV associated with corn occurred in the Piedmont Region of northern Italy, which is next to the Lombardy Region (Lomonaco, 2012. EPIDEMIC CLONES OF LISTERIA MONOCYTOGENES: DETECTION, TRANSMISSION AND VIRULENCE. In, Listeria Infections: Epidemiology, Pathogenesis and Treatment). This needs to be discussed in the context of the results presented and the reference for this outbreak (Aureli et al., 2000) needs to be also included in the manuscript.

This has been added in the discussion along with the references.

8. The subheaders in Table 2 should read “Number of cases with underlying condition” and “Isolate/Subtype data”. Plus explain below this Table why PFGE clusters 1 and 10 were not included.

This has been done.

9. On Figure 1 indicate “percent of isolates by serotype” and “total number of isolates” on the left and right x-axis, respectively.
This has been done.
10. On Figure 2, put PFGE Cluster on far left, followed by code, year, serotype, ST (CC) and epidemic clone. This will make the order consistent with that in Table 2. Also, indicate if ECV is present. Also delete title at bottom.
This has been done.
11. On Figure 3, change 38 to 101 and recheck all other STs to make sure they are the correct clonal complex.
This has been done.
12. Rather than using the term “molecular subtype clusters”, the authors need to be specific and call them PFGE clusters throughout ms. Also, if ST, CC or EC are used then different clusters result, as mentioned above this needs to be discussed – how and why are these clusterings (using different molecular markers) different? What do they mean? Which markers are more relevant/accurate/useful for epidemiologic investigations? This needs to be included in the discussion section.
The definition of clusters has been modified. About the remaining issues, I am combated between the suggestions to add and those to cut. I hope the present version could be more adequate from this point of view.
Minor Essential Revisions
1. The manuscript needs a thorough review by someone fluent in correct English grammar to clarify meaning in many, many places
This has been done.
2. In many cases the authors say there is a difference, but then say it is not significant (see bottom of page 9). Either delete these statements or say there was no significant difference.
These sentences have been written in a more straightforward way.
3. Need references to support statements like, “Lombardy an Italian region accounting for 16% of the nation’s total population, is notifying 55% of the listeriosis cases” Where did the data come from to allow calculation of these values? What time span are the authors referring to? How many outbreaks in total were reported in Italy during that time span?
This has been done.
4. Need to discuss why PFGE Clusters, STs, CCs and epidemic clones don’t always agree (see Table 2).
Please, see above Major revisions 12.
Discretionary Revisions
1. Paragraphs following the first paragraph need to be indented 2. Need to clarify the time period (span) in question in many places 3. It would help to know what national and regional Italian Health Agencies were responsible for investigating listeriosis cases and detecting outbreaks in the Lombardy Region of Italy and what their different roles were during the period in question 4. Poor word choice in many places, need to choose the right word 5. How many total cases of listeriosis were there by year throughout the period in question?
I have tried to meet some requests.

Reviewer 2: Brita Bruun

Reviewer's report:
The manuscript contains a wealth of information on the population dynamics of Listeria monocytogenes from invasive infections in Lombardy, Italy, that is bound to be of interest to specialist in this bacterium’s epidemiology. The aims of the study are clearly stated and the
methods used are appropriate and adequately described, if one disregards the need for improving the English. Limitations of the study are given, and the conclusions are supported by the data. The title and the abstract convey the results correctly.

MAJOR COMPULSORY REVISIONS
General:
1. The manuscript is far too long and must be shortened by 25-30%, which in part can be achieved by avoiding repetition of data in the text and in the tables plus figures. This has been done.
2. The English is understandable, but needs a good deal of revision before being publishable. Some of these will be noted below, but I recommend that the authors seek help as it is impossible for me to list all my specific suggestions. I have tried to achieve such objective.

RESULTS
Paragraph 2:
a) As I read the text there were 118 non-pregnancy cases with 118 isolates and 15 pregnancy cases with an isolate from both mother and child in one case, giving 16 isolates (118 + 16 = 134 isolates). Were the "double" pregnancy isolates different from each other? If not, shouldn't the case only be represented by one isolate?
Yes, this is true. But the isolate wasn’t removed, because we thought that a single isolate doesn’t influence prevalence of types, associations and conclusions we have achieved.
b) The text states that 56.2% of the non-pregnancy cases were older than 65 years, while Table 1 has 64.4% of cases being more than or equal to 65 years. This needs clarification.
I have checked the numbers and revise them. Sorry!
c) Text states that 96.3% of isolates from non-pregnancy cases derived from blood and/or CSF, while Table 1 has 76.3% + 20.3% (=96.6%) of isolates derived from blood and/or CSF. This needs clarification.
I have checked the numbers and revise them. Sorry!

Paragraph 3: The text states that the number of cases with serotype 1/2a increased from 7 in 2006 to 23 in 2010, but as far as I can tell from Table 1 it rose from 19 in 2006 to 34 in 2009 and then fell to 24 in 2010.
The legends of axes have been revised. So, the figure should be easier to interpret.

DISCUSSION
Paragraph 4. As far as I can count, Fig. 3 shows that there are 11 MLST clonal complexes with 121 isolates; the text says "PFGE and MLST ..... gave very consistent results allowing to include 112 out of the 134 isolates under study into 12 clusters containing three strains." Do the authors mean "PFGE and MLST ..... gave very consistent results allowing to include 112 and 121, respectively, out of the 134 isolates under study into 12 and 11 clusters containing three strains"?
The discussion has been revised and considerably cut, so the above sentence is no more present..

MINOR ESSENTIAL REVISIONS
RESULTS
Paragraph 5: The sentence "12 pulsotype clusters, including three or more isolates, were identified (Fig.2)." is repeated as part of the contents of a sentence appearing one line below. The sentence has been removed.
Paragraph 6: The section starting with "The 1/2a/CC38 isolates" and ending with "affected subjects older than 65 years." is superfluous, as the information is given in Table 2. It is also unnecessary to list the various underlying conditions/diseases. Furthermore, the text states that
27 (84%) involved subjects older than 65 years, while Table 2 has that 26 persons were older than 65 years. This has been revised, cut and modified according with the suggestions of the reviewer.

DISCRETIONARY REVISIONS
1. Using the term "MLST types" instead of "sequence type" is preferable, as is "typing" instead of "subtyping".

RESULTS
Paragraph 4. Non-significant serotypes differences in relation to age and fatality rate could be deleted (shortens the manuscript).
Paragraph 6. The patient demographic and clinical information whose isolates belong to cluster 11 should be limited to possible differences between this cluster and the other clusters (shortens the manuscript).

I have tried to meet some requests. The title has been changed according to the suggestions of the reviewer 1, who asked us to be more specific and indicate the conclusions reached.

Reviewer 3: Benjamin Silk

Reviewer's report:
This is an excellent summary of surveillance data on invasive listeriosis cases and L. monocytogenes isolates from a populous region of Italy. In particular, the authors have demonstrated considerable expertise in the molecular epidemiology of listeriosis. However, the report is overly long and could be condensed to improve readability. While valid, conclusions related to the importance of outbreak detection and investigation are not supported by the data because no outbreaks were detected in Lombardy, Italy during the study period.

Major Compulsory Revisions
1. The report is overly long (approx. word count= 3,900) and could be condensed to improve readability. The background (~600 words) and discussion/conclusions (>1,700 words) might be best places to remove extraneous content. Also, redundancy in reporting data in the discussion, when similar data have already been described in the results, should be reduced.

The manuscript has been revised and cut thoroughly.

2. While valid, conclusions related to the importance of outbreak detection and investigation are not supported by the data because no outbreaks were detected in Lombardy, Italy during the study period. Instead, I expected more of a synthesis to provide the key points on this summary of the molecular epidemiology of listeriosis in Italy.

3. As a related comment, the authors report that no outbreaks were detected during the study period. However, there is no information presented on what is done to identify or investigate clusters. In other words, it would be interesting and useful to know more about the statistical or analytic methods that are applied for cluster detection and/or epidemiological methods for cluster investigation (e.g., patient interviews on food exposures). Otherwise, the reader is left wondering how it is possible that no outbreaks were detected, and especially whether outbreaks were missed.

2 and 3. Similar concerns have been raised by the reviewer 1 (Dr. S. Knabel). It is quite reasonable to ask us to add this information, or rather, to embed our laboratory-based findings into the surveillance and epidemiological investigation activities conducted by the regional and local public health authorities. However, as it has been discussed above (please, see the answer to Dr. Knabel), our study is a retrospective epidemiological study on cases of listeriosis occurring during a five-
year period of enhanced surveillance. The “enhanced” surveillance is described in the Methods. I understand that our system is to some extent light-years distant from that of US, but this is the state-of-art in Lombardy, that is among the best in Italy, as the notification rate shows.

Minor Essential Revisions
1. Overall, the quality of the writing in English is good. But it may still be beneficial for a writer/editor with English fluency to review the manuscript. For example, the discussion uses the word attitude incorrectly in two places. Is the intended word aptitude? Another example is in the third paragraph of the introduction, where the first sentence would read better with a change in verb tense “…notification of listeriosis has been mandatory since 1993.” A third example is the fifth paragraph of the introduction, where the first sentence needs to be rewritten to improve sentence construction.
This has been done.
2. Some results report means with an accompanying statistical measure of variability. For example, the second paragraph states “…mean age of patients was 64.7 ± 15.1 years. This measure of variability should be defined in the methods above.
This has been done.
3. In the results and table 1, the terms case fatality rate, all-cause fatality rate, and crude fatality rate are used interchangeably. If they are the same measure, a single term should be selected for consistency. If they are not the same measure, some explanation of the difference would be helpful.
This has been done.
4. Many readers may print the article in black and white, which makes the color schemes for the legends of the figures very difficult to discern. It would be very helpful to reconsider using shades of colors that are amenable to black and white print. Also, I did not see where the legend for figure 1 defines percentages versus number of isolates.
This has been done.

Discretionary Revisions
1. It seems reasonable to assume that invasive listeriosis predominately affects immunocompromised individuals and the elderly everywhere, not just in European countries. Accordingly, the first sentence of the second paragraph in the introduction could be revised.
This has been amended.
2. In the fifth paragraph of the introduction, the statement about ‘electronic probability of typing data’ needs clarification. I see MLST as advantageous because it is becoming a global standard, which aids in broader comparisons of L. monocytogenes isolates internationally.
The statement says “portability” (no probability).
3. The methods section has five subheadings that could be merged into one broader subheading, such as “Isolate characterization.”
This has been done.
4. In the second to last paragraph of the results, the sentence “No epidemiological link was detected…” could start a new paragraph since it begins an idea that is separate from the preceding sentences in the paragraph.
5. In the second paragraph of the discussion, which considers reasons why outbreaks are difficult to detect, the statement “infrequent clinical manifestations vs. a likely frequent exposure” needs clarification to better convey the meaning.
6. In table 2, the title includes “Only the clusters containing more than three isolates are included,” which would be more appropriately placed below as a footnote.
4-6. All these suggestions have been accepted and the manuscript revised accordingly.
Reviewer 4: Hendrik Cornelis den Bakker

Reviewer's report:
General comments:
This manuscript describes the molecular epidemiology of Listeria monocytogenes in the province of Lombardy, Italy. The report of interest for the Listeria community because is it gives a good overview of L. monocytogenes subtypes in a particular geographical region over a specific time period. The methods and results are appropriately described, however the discussion could be shortened and throughout the manuscript minor language errors can be found, which sometimes make part of the text unintelligible. Unfortunately, the current manuscript lacks line numbers, which makes it hard to point out specific errors in the text.

Major Compulsory Revisions:
Please have a native speaker with knowledge of the subject read through the manuscript, it currently contains multiple minor language errors, which make some parts of the manuscript unintelligible.

I have tried to do this. I hope the present version could be more reader-friendly

Minor Essential Revisions
Please provide line numbers in the revised manuscript. Without line numbers it is a lot of work for the reviewer to point out specific parts of the manuscript that need revision.

Line numbers are no required by the Author’s guidelines, but they have been included in the revised version.