Author’s response to reviews

Title: Traceability and distribution of Neisseria meningitidis DNA in archived post mortem tissue samples from patients with systemic meningococcal disease.

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Author’s response to reviews:

Dear Professor Cecilia Devoto

Editor of BMC Clinical Pathology

We hereby submit the revised manuscript entitled “Traceability and distribution of Neisseria meningitidis DNA in archived post mortem tissue samples from patients with systemic meningococcal disease.” for your consideration and possible publication as a Research article in the BMC Clinical Pathology.

We gratefully acknowledge the comments raised by the reviewers, and think that their remarks have improved the manuscript significantly. The comments have been addressed below consecutively, and corrections are also highlighted in the manuscript in red. A revised Table 1 is included the manuscript while the “Table 1 before revision” is sent as an attachment to the manuscript. We hope that the Editorial Board will find the revised version acceptable for publication BMC Clinical Pathology.
Reviewer 1:

TO THE AUTHORS

Comment:

Merits.

The paper is within the scope of the journal. The study focused on a relevant issue for pathology and it underlines the usefulness of fresh frozen collection for further testing in case of systemic meningococcal disease. The research is also well designed in the molecular study and this is shown also by the use of a DNA-degradation marker (HBB gene).

Response:

We highly appreciate the positivity with which the reviewer received our manuscript!

MAJOR COMMENT

Comment:

Critique.

Collected samples are few (seven) even if the numbers and type of organs are consistent. The authors should better explain that the negative results in the two 28 years old specimen could be a false negative result due to the degradation, i.e. non detectable DNA.

Response:

We agree with the reviewer that the most likely explanation of the negative N. meningitidis PCR result in the brain tissues which contained meninges is degradation of N. meningitidis DNA. We know that these patients had high levels of N. meningitidis LPS in the cerebrospinal fluid as indicated in Table 1. The levels of LPS closely reflect the level of N. meningitidis DNA (ref 11). We assume that meningococcal DNA in human tissues is degraded in parallel with human DNA. The pace of degradation of human HBB DNA in this study is given on page 12 (Evaluation of the quality of isolated DNA from FFPE and FF tissue) and Fig 1. We have explained this on page 17 and 18.

MINOR COMMENTS

- p.5 line 28: the acronym should be moved in p.4 line 15 where it is cited first

- p.15 line 1: since two papers were cited, add the "Guarner et al in the text"
Response:

We thank the reviewer for noticing information that is lacking, and have now revised this in the manuscript, corrections are highlighted in red.

Reviewer 2:

MAJOR COMMENTS

Comment 1:

How did you specify the localization of bacterial distribution of N. meningitides or the bacterial DNA in the autopsy setting?

Response 1:

The patients included in this study were all diagnosed to have meningococcal disease based on presence of N. meningitidis in blood or in CSF (culture and PCR methodology) in addition to other clinical definitions. The different FFPE tissues were, after preparation, characterized by microscopy and described based on hemorrhage, edema and fibrin thrombi. Intact meningococci were not observed. The distribution in the different organs was based on PCR methodology.

Comment 2:

How did you rule out the contamination or false positivity of N. meningitidis DNA or the bacterial DNA in the autopsy setting?

Response 2:

We understand the reviewer’s concern. The autopsies were performed according to the routines at the Department of Pathology including procedures to prevent contaminations from other bacteria. N. meningitidis deaths are very rare (several years between each case) in the hospitals involved. Hundreds to thousands of autopsies have been performed with proper cleaning procedures between two autopsies with meningococcal infection. Contamination from a previous patient is ruled out owing to routine cleaning procedures and long interval between cases.

Comment 3:

How did you take tissue samples during the autopsy setting? Did you use sterile equipment like sterilized surgical knives?
Response 3:

Routine autopsy do not normally use sterile equipment. However, when specimens are to be investigated by microbiological analyses, sterile equipment is used. We have now included a brief procedure on how the autopsies are performed in Material and methods.

Comment 4:

How did you prevent the contamination from the affected organs to the non-affected ones?

Response 4:

Tissue specimens from the different organs (heart including endocard and epicard, lung, liver, spleen, kidney) were put into a container with 4% buffered formalin solution for 6-48 hours. The whole brain was stored in a separate container and fixed in unbuffered formalin for at least 3 weeks. After fixation the tissue specimens were embedded in paraffin, sliced in 4µm thick sections and stained with Hematoxylin and Eosin (H&E) for microscopic examination. The paraffin blocks were stored at room temperature.

A clean microtome knife, washed in 96% ethanol between each block, and sterile tweezers were used when cutting sections from the FFPE tissue blocks. The first sections were not used in order to avoid surface contamination. The tissue sections were put into sterile PCR-clean micro tubes.

Our research group has worked with PCR techniques since late 1980ies. We are very focused on contamination using these sensitive techniques and have always taken the necessary precautions which are reflected in a large number of publications (such as Ref 11).

Isolation and detection of Neisseria meningitidis DNA from these different tissues gave variable and reproducible results. Our findings suggest that tissues from the same patient were invaded by N. meningitidis at a variable degree. Certain tissues contained very low levels of Neisseria meningitidis DNA whereas others levels were high.

We interpret these variable results as a support of our procedures. Contamination with N.meningitidis DNA we assume would lead to a more even concentration of Neisseria meningitidis DNA in all organs. The negative controls in each experiment including the tissues of a patient dying from S. pneumoniae infection were negative.

Furthermore, we did not found any evidence for a positive N. meningitides DNA in any FFPE tissues from our negative control (pneumococcal infection).

Brain tissue from three patients with meningococcal septic shock was negative for N. meningitides DNA. We know that these patients have a very low level of N. meningitides DNA
and N. meningitides LPS in cerebrospinal fluid (ref 4,5,11). These negative findings also indicate that there was no general contamination from the cutting of incisions.

Most importantly, our results are in close accordance with the results obtained in ref. 21 (Hellerud, B.C., et al., Massive Organ Inflammation in Experimental and in Clinical Meningococcal Septic Shock. Shock, 2015. 44(5): p. 458-69). The publication document the presence of N. meningitidis DNA in various organs of patients 3,4,5 in this publication applying the same PCR method but performed by another research group in another laboratory. The presence of N. meningitidis DNA in the different tissues was accompanied with a massive increase of major proinflammatory molecules including (TNF, IL-1b, IL-6 and many chemokines) as measured by immunoassays. Controls including lethal cases of pneumococcal sepsis and sudden infant death syndrome (SIDS) were N. meningitidis DNA negative and had a much lower levels of inflammatory mediators in the tissues. To us this rules out tissue contamination of N. meningitidis DNA as a likely explanation of our present results.

Lately, we have also performed gene expression studies in the same tissue samples that were used for N. meningitides DNA quantification. The results show significantly increased inflammatory responses in the tissues with high levels of N. meningitides compared to tissues with low or no presence of N. meningitides (Manuscript in preparation).

Comment 5-1 :
How did you deal to fix the tissues or the organs? Did you separate them to prevent the contamination?

Response 5-1:
These matters are described in the response 4.

Comment 5-2 :
The authors examined the genetic method as quantitative real-time PCR for N. meningitides which are relatively sensitive and specific than classic microbiological methods including bacterial cultures. They concluded that the high levels of N. meningitidis DNA can attribute the development of shock, probably contributing to the multiple organ failure.

Post-mortem microbiological detection has been discussed in several studies (F.J. Roberts, PMID 10052543) (G.C. du Moulin, PMID 3888815). Heart blood and cerebrospinal fluid are the most useful substances for post-mortem bacteriological cultures (M. Tsokos, PMID 12935728). False-positive results caused by contamination have been obtained during lung tissue cultures.
Response 5-2:

Neisseria meningitidis in Norway is highly sensitive to benzylpenicillin and chloramphenicol, the two antibiotics used to treat these patients at the time. Blood cultures are negative shortly after initiation of the treatment as are the organ cultures at post mortem examination. Detection of viable bacteria 12 to 24 hours after initiation of antibiotic treatment is not an option.

In this study, only FFPE and FF tissues were used and our results indicate that our procedures prevent contamination between organs.

Comment 5-3 :

Both the microscopic findings and the classical culture method have been used for diagnosis of true infection. There is a long history of improving the culture method and developing new detection methods for the infection. Recently, it has been demonstrated that PCR and serology test are much sensitive than culture for detection of sepsis. In the setting of the sterile blood samples, the qPCR is one of the most sensitive and specific methods for the detection of bacteremia or sepsis besides the blood culture samples (Ziegler I, PMID 27997618). However, we need to distinguish the N. meningitidis DNA which localizes in the tissue parenchyma or the blood of organs and the inside of vessels. I would like to recommend you not only showing the distribution of N. meningitidis DNA but also the pathological evidences of each tissue sample on microscopic findings.

Response 5-3:

Pathological evidence of each tissue sample based on microscopic findings by an experienced pathologist is now included in Table 1.

Comment 6:

We need to consider the septic changes in the microscopic findings of the autopsy. In the Table 1, you mentioned clinical characteristics; microscopically examination of tissue. We need to consider the septic changes such as neutrophilic infiltration, necrosis, or hemorrhage in the each organ (Sebastian Lucas, http://dx.doi.org/10.1016/j.cdip.2007.06.001).

Response 6:

We thankfully value the suggestion from the reviewer, and have rewritten table 1 in the revised manuscript.
Comment 6-1:
Fibrin thrombi in 1-Serogroup B and 4-Serogroup B. Did you mean that the fibrin thrombi locate in glomerular capillaries or other vessels?
Response 6-1
The fibrin thrombi were located in the glomeruli capillaries.

Comment 6-2:
Neutrophils in alveoli in 2-Serogroup B. Could you distinguish pneumonia and septic change in the alveolar septae or alveolar capillaries?
Response 6-2
A localized area with atelectasis, karyorrhectic cells, some neutrophils and small hemorrhages is seen in one of the lung sections, possible an early ischemic necrosis. This morphology is not typical for bronchopneumonia or septic microabscesses.

Comment 6-3:
In 5-Serogroup B, you mentioned “normal”. Do you mean that there are no specific changes associated with sepsis in the case? It may be difficult to say the cause of death associated with sepsis in the case.
Response 6-3
We understand the reviewer’s point of view. Yes, we meant there are no specific changes associated with sepsis in the case. We have revised this in Table 1. The cause of death due to sepsis is based on the detection of Neisseria meningitidis as well as LPS in blood and spinal fluid. The patients develop treatment resistant septic shock with persistent hypotension and increasingly cardiac failure leading to a terminal arrhythmia. Fifty percent of patients with this clinical presentation die within 12 hours after hospital admission explaining lack of microscopically evidence of inflammation in many patients.

Comment 6-4:
In 1-Serogroup B and 3-Serogroup B, what do you mean by “Shock kidneys”? Do you mean acute tubular injuries or acute tubular necrosis?
Response 6-4

The term “shock kidney” is based on a macroscopic evaluation of an experienced pathologist after section of the kidney revealing a pale cortex and dark-red medulla. Microscopically the vessels in the medulla are dilated and blood-filled.

Acute tubular necrosis is often difficult, sometimes impossible to differentiate from pure post-mortem changes.

MINOR COMMENTS

1. Abstract. In the page two, line 14-15, please use the plural form “lungs” or “kidneys” same as the page seven, line five.

2. Background. In the page three, line 18, please check the spell, “petechiae”.

3. Material and methods. In the page six, line 4, 8, 16, 22 and 23, please use the abbreviation from “no” to “No.”, and also seen in Figure legend in Fig.2.

4. Material and methods. In the page six, line 8, and 16, please use the singular form “patients” to “patient”

5. Table 1. In 6-Serogroup B and 7--Serogroup B, please check the spell, “pus”.

Response:

We thank the reviewer for correcting grammatical errors and spelling miss. We have now changed this in the manuscript; corrections are highlighted in the manuscript in red.