Author’s response to reviews

Title: Heavy Metal Exposure and Nasal Staphylococcus aureus Colonization: Analysis of the National Health and Nutrition Examination Survey (NHANES)

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Author’s response to reviews:

Environmental Health Editorial Board:

We would like to thank you for your consideration of this resubmission. We also thank the reviewers for their thoughtful review of the original manuscript, and for the insightful comments and critiques; we have addressed them in the resubmitted manuscript and feel based on these suggested revisions the manuscript is much improved. Below is a point-by-point reply to reviewers’ comments. We have also attached both a clean and track-changed version of the manuscript to support review of these changes. Again, we thank reviewers for the careful attention to details and review of this work.

Reviewer 1’s Comments

R1 Comment: The authors assume a level of familiarity of NHANES and the nature of the purpose of the study and nature of the variables collected. The paper would be strengthened if more explanation of the background of the NHSANES survey, setting, cohort descriptives and population could be briefly added.

Response: Further description of data collection has been added, and a general description of the cohort size and sampling frame is included in the first paragraph of the methods. Further description of the nature and purpose of NHANES has been included in many previous publications, and is not the main purpose of this study, therefore we added another citation to the NHANES website which gives a very detailed description of the nature and purpose of the study. Table 1 gives a more detailed description of demographics within the analytical sample.
R1 Comment: Good definitions of the variables and explanation of the models used to calculate risks. More detail of the variable selection and omission, both a-priori and post-priori is needed to explain and justify the model building process. Why were some variables left out on purpose, e.g. smoking in the Lead models?

Response: Models were re-examined, and smoking has been added to the lead model. The model building process is explained in more detail in the methods section.

R1 Comment: Throughout the paper there are strong assertions made for a strong and consistent association found. This conclusion was made on the evidence presented that seems more like mixed findings and perhaps weak signal strength associations that are found after multiple testing and pre-defined models. Did the authors consider the effect of making multiple comparisons, that you would find 1/20 results as significant (type 1 error) and test the effects of adjusting the p value appropriately (e.g. Bonferroni correction or other)? Model choice and inclusion of variables also seemed to be very prescriptive. Did you conduct a sensitivity analysis to assess the effects of other variables and confounders?

Response: Thank you for your comments and critical review. This analysis tests two hypotheses: that Pb (1) and Cd (2) exposure would both be associated with an increase in MRSA colonization by co-selecting for metal and antibiotic resistance and a decrease in MSSA colonization by reducing the abundance of metal and antibiotic susceptible bacteria. To test these, 2 comparisons were made for each hypothesis. Multiple models were shown to illustrate the effect of confounding variables. When using 2 comparisons per hypothesis, multiple comparison corrections are not necessary. Variable choice is explained more thoroughly in the methods section. A priori selection of variables was used to create parsimonious models that did not include variables that were spuriously correlated.

R1 Comment: The authors use frequent references to 'Pb/Cd exposure' when referring to the blood lead/cadmium levels measured in study participants. It is (probably) unknown what the level of environmental exposure resulted in the blood level, or what 'exposure' was when the samples were collected. The blood concentration would reflect the body burden and intake/uptake of the metal, but not necessarily the concurrent environmental exposure. Some reference or explanation of this fact needs outlining, and perhaps reference to 'blood Cd/Pb levels' would be a more accurate description/label.

Response: Thank you, we thoroughly agree with this comment, Exposure' in this context has been changed to 'blood level'.
R1 Comment: It would also be beneficial to add a description of the half-life of Pb and Cd in blood and some thoughts on when and how the metal exposure could have occurred; i.e. how and what the blood levels actually represent. Also further explanation of how colonisation of MRSA/MSSA occurs. Can carriage vary (come and go)? Once you acquire it does your status change? Do we know if it is recently required etc? Can you be colonised by both MRSA and MSSA at the same time, at different body sites, and/or swap status? This may help to explain the associations between the two (or is this a limitation of the study if unknown?).

Response: Requested details have been added to the discussion section including information on half-life of both lead and cadmium levels in the blood and description of MRSA and MSSA colonization. Additional text has been added to explain the potential complexity of associations observed in the study. In brief “it is not clear whether acute or chronic exposures have a greater influence on the association with S. aureus, as source and length of S. aureus colonization cannot be determined.”

R1 Comment: The authors list a few limitations of their study, and it is an important first step to exploring the relationships, but there are other limitations that were not addressed, e.g. could there be alternative explanations for the associations found? Other (residual) confounding, interactions, biases or chance? What might these be? Was the analysis limited to the format of NHANES outputs? What other information would be useful to collect to explore these associations (e.g. current smoking status, SES, occupation, etc.)? What other information do we need to collect to further explore this issue?

Response: Thank you again for pushing the discussion to be more detailed. Additional consideration of residual confounding has been added to the limitations in the discussion section.

Abstract: --

R1 Comment: Sample size is missing.

Response: Sample size was added to the methods section of the abstract.

R1 Comment: The wording suggests cause and effect, e.g. 'both metals were protective against MSSA'. No causal relationships are proven, and the associations were often weak or not-significant. The wording around the strength of the associations in the whole manuscript could be toned down.

Response: Agreed, language throughout the manuscript was changed to highlight associations instead of causality.
R1 Comment: Line 26: Can the authors explain what 'co-select' means in this application?

Response. We agree this is a difficult term not often used in EH research, but known and used regularly in infectious disease epidemiology and in previous work describing how metals may confer antibiotic resistance. Text within the abstract was edited to highlight the main point that heavy metal exposure may be promulgating antibiotic resistance. We added details to the main body of the text to describe the many potential mechanisms of co-selection but hesitate to add this level of detail in the abstract in order to ensure readability. We would be happy to add text now in the main body regarding co-selection into the abstract if necessary.

R1 Comment: Results- add sample size. Some sweeping statements are given regarding metal concentrations found and MRSA. On further examination these differences between the GMs are not statistically different, so either the language needs to reflect this or p values given.

Response: We agree with the reviewer this was missing, sample size has been added to the methods. Language has been altered to emphasize that GMs are not statistically different despite overall significance in p for trend analyses.

R1 Comments: 'both metals were protective against MSSA': This assumes causation. A dose response relationship was shown, but with adjustment for confounders, there is little difference in the ORs of the quartiles (CIs overlap).

Response: Agreed, language changed to highlight associations instead of causality.

R1 Comment: Conclusions: the authors refer to 'current' population exposure when in fact the samples were taken 13-16 years ago and environmental exposures and indeed blood Pb/Cd levels may have changed during this time. How do the authors know that the metal exposures and colonisations was concurrent?

Response: The word current was changed to general, to imply that study participants were not necessarily occupationaly exposed to either metal. The metal exposure and colonization were concurrent because samples used to assess both were collected at the same time. Further explanation of this has been added to the methods section of the paper.
Background:  --

R1 Comment: line 105: Explain what 'co-select' means in this context.

Response: We agree, the paragraph prior to this line gives a detailed explanation of co-selection. Further details were added to support understanding of co-selection and its importance in antibiotic resistance.

R1 Comment: Line 112: could the authors explain (somewhere) what literature searches they undertook to make this statement (search strategy or evidence)? This could be outlined as a strength of the study in the discussion.

Response: Because this study is not a review, we feel that a description of the literature search protocol is not necessary. Language in this sentence was altered to indicate a greater level of uncertainty.

R1 Comment: Line 116: A 'health-care' acquired infection? Not just hospitals?

Response: The word hospital has been changed to health-care.

R1 Comment: Line 123- please add a reference to this sentence or assertion.

Response: A citation has been added.

R1 Comment: Line 133-135: This is a causation argument and authors suggest a sequence of events that exposures causes colonization. However, the authors later on in the paper suggest that the order is unknown (fig1), so this is not consistent.

Response: Language in this sentence was changed to de-emphasize the causal pathway, and make it more consistent with the discussion and Fig. 1.

R1 Comment: Line 137: Refers direct to metal exposure and associations with colonisation when in fact the relationship is between blood lead/cadmium levels measured and concurrent colonisation (see comment before). We assume both samples were taken at the same time?

Response: Language was changed to indicate blood measurement of Pb/Cd, and that S. aureus colonization was measured concurrently with the blood metals.
R1 Comment: Line 138: define adults.

Response: The word adults was used in error. "Adults" has been replaced by "residents".

Methods: --

R1 Comment: Data sources: How was the data extracted, by whom and how? Can you give more details about NHANES as some readers maybe unfamiliar with this survey? How were the participants selected and recruited? More detail about when and how the biological samples were taken would help (e.g. concurrent - on the same day/clinic visit?). The reference refers to an analytical guide (33), not necessarily giving details of the background to the survey and how the sample population / cohort was selected and recruited. Are they representative? What were the waves of the survey - does this matter?

Response: See response to earlier comment about familiarity with NHANES. The paper explicitly states that survey data was from the 2001-2004 waves.

R1 Comment: Line 150: Table 1 suggests age was 0+.

Response: Table 1 has been changed to indicate age 1+

R1 Comment: Line 151-2: Was ethics required? If publically available can you give a reference for this (url?)

Response: Ethical review is not required for secondary analysis of publicly available, de-identified data sets, as indicated by this sentence in the text. A sentence was added, with url, to indicate where questionnaires and data sets can be found.

R1 Comment: Line 165-167: Is this a standard assumption made for colonisation? Can this be referenced?

Response: If a person is not colonized by Staphylococcus aureus, they cannot be colonized by S. aureus that is resistant to methicillin. This is a statement of logic, thus no reference is necessary.
R1 Comment: Line 176: Why was smoking not included in the lead model? Lead is known to be present in tobacco smoke and cigarettes. What was the method of the choice of variable to include in the models and why were some variables pre-defined and left out of models? Did the authors consider backwards or forwards stepwise model building? Did this have an effect on the risk estimates?

Response: See previous comment about model variable selection.

R1 Comment: Line 177: Was it possible to separate current smokers and ex-smokers, as this could explain both the exposure and outcome variables? If this data was not available, it should be discussed as a limitation of the study?

Response: The smoking variable was re-categorized to separate current and former smokers. An explanation of smoking categorization has been added to the methods section.

R1 Comment: Diet data: Line 179 onwards. It is not clear how the different diet data were calculated for the different metals. Can more explanation of what was included for each model be given? Was green leafy vegetables separated or used as a variable? What differences were considered? Did this make a material difference?- line 188- can you give a reference for the food codes?

Response: The second paragraph of the "Confounder Measurement" section explains that total grams Vitamin C, Iron and Calcium were included in the Pb model, and why, and that total grams fruit and vegetable consumption was included in the Cd model and why. The "statistical analysis" section also explicitly states which variables are in each model. A reference was added for the food codes.

R1 Comment: Statistical analysis: Line 199- explain what is meant by a DAG? How did this affect variable choice? It seems that only a-priori confounders were considered in the models. What was the effect of other variables? Were the effects of other confounding and effect modification considered? Was a sensitivity analyses undertaken? How was the choice of different dietary options optimised for each model?

Response: A DAG is a model that supports identification of causal pathways in models where many variables may be included. Use of Directed Acyclic Graphs and choosing covariates a priori based on subject knowledge was used in selecting variables for this model and standard for many if not all epidemiologic investigations of complex pathways. The goal is to create the most parsimonious model possible without overadjustement and potential bias. We chose dietary data
based on known confounders with respect to exposure and response. The data come from the
NHANES food frequency questionnaire and categories of response were consistent with data
analyzed and available through standard coding by NHANES.

Results. --

R1 Comment: Descriptive statistics need to include some background to the population cohort,
numbers sampled, how recruited and how representative the sample is to the population. Are the
differences in colonisation rates between the groups real? Do the CIs overlap? Can you provide
some statistical test of real differences between groups?

Response: Background on the population is included in the methods section, and is not repeated
in the tables because the methods section can be easily referenced. The years of data collection,
and total sample number have been added to the table legend. P-values have also been added to
the descriptive statistics.

R1 Comment: What levels of Pb and Cd were found? Mean, min, max, GM etc? What were the
levels of the quartiles? How do these levels compare to other populations? Are the results
transferable to other populations?

Response: Geometric mean Pb and Cd values are given with confidence intervals in table 1.
Quartile ranges for both metals were added to the methods section.

R1 Comment: Table 1: Define the years in the title of the table. Define the % given in the
headings (i.e. row %ages?). It would be good to see the breakdown of the sample into the
categories, i.e. by adding a totals column and add % of the columns (e.g. % of the sample in each
age group, % of males to females, % ever to never smoked, etc). There are discrepancies with
the 0-17 age group with the text of a minimum age of 1 year.

Response: Years of data have been added to the table description. "Row" has been added to
column headers to specify type of percent shown. To avoid cluttering the table with too much
data, we decided to include only row % instead of row and column %. We feel that row % is
more important to display as it helps to highlight the differences in prevalence of MRSA and
MSSA colonization between each level of each variable. However, totals for each column are
included at the top of the table, so column percentage can be easily calculated by the reader if
interested.
R1 Comment: Table 3: What are the units of these measurements (grams?)? Use label: fruit and vegetables.

Response: Unit of measure is indicated in the row header. Veggies has been changed to vegetables.

R1 Comment: Line 231: 'Exposure to Cd is associated with decreased odds of MSSA carriage…' but model 3 (Table 5) shows that by adding fruit and vegetable consumption, then the OR are not significantly different from 1. Can you explain this?

Response: Because the upper confidence limit for the 4th quartile in Model three is just above 1, we think this effect is still worth noting, however we adjusted the language in line 231 to indicate marginal association, rather than significant.

R1 Comment: Table 4: what was the effect of adding smoking to the Pb models?

Response: Smoking has been added to the Pb model. Results of previous analyses did not change after adding smoking to Model 2. Associations comparing odds of MRSA between Q1 and lead remain unchanged, inverse associations with cadmium levels continued to be significant when smoking was added to both models 2 and 3

Discussion. --

R1 Comment: Some of the assertions made assume that the differences found are real. Is there any unexplained residual confounding, is the result due to biases or chance? These points should be discussed and if not addressed listed as limitations of the study.

Response: See previous comment about residual confounding.

R1 Comment: Line 240: I do not wholly agree with the statement that increasing Cd exposure was associated with MRSA colonisation. In the fully adjusted model (3) in Table 4 the OR do not increase and are only significantly reduced for the highest quartile.

Response: Analyses have been updated. The fully adjusted model shows a significant decrease in odds of MRSA exposure with Cd levels above the 1st quartile.
R1 Comment: Line 245 onwards- what searches were carried out and on what databases to make this assertion? How complete were the literature searches?

Response: As previously mentioned, search criteria are not necessary as this paper is not a literature review. The phrase 'to our knowledge' is included to indicate that we may not have found every relevant publication in our search.

R1 Comment: Line 264: 'over time'.

Response: The word has been changed.

R1 Comment: Line 265- can the pattern be explained? (relates to point above)

Response: An explanation for the pattern is given two sentences later.

R1 Comment: Line 273-275. What Cd levels does this relate to? What is high? How is green leafy vegetables measured in this study? Would it not be the case that both Cd and Pb be a factor via this route? Could you explore the datasets in this study to test this?

Response: Explanation of vegetable measurement is given in the methods section. As lead is not associated with vegetable consumption, Cd is most affected by this interaction. The word 'high' has been removed.

R1 Comment: Line 284-285. Perhaps weak and not consistent evidence is presented. Many OR were not statistically significant and there may have been multiple testing issues and residual confounding not explained. There seems to be some pattern that suggests a possible association.

Response: Language has been revised to reflect a weaker association than previously asserted.

R1 Comment: Line 293- suggest being specific to 'nasal' colonisation, as colonisation of other parts of the body have not been explored.

Response: The word 'nasal' has been added.
R1 Comment: What other limitations apply to the study? Could anything else explain the associations found? Do you think there is residual confounding or interactions? Was current smoking status explored, or the effects of household second hand smoke, occupation, etc?

Response: See previous comments about residual confounding and smoking.

R1 Comment: Line 307- can you please explain what is meant by 'reverse causality' in this context?

Response: An explanation of reverse causality in this context has been added.

R1 Comment: Line 312: Explain MDROs. Also add to list of abbreviations - line 325.

Response: MDROs has been removed and replaced with 'antibiotic resistant bacteria'.

Reviewer 2's Comments

R2 Comment: The conclusions and recommendations do not follow from the collected data nor from the stated aims of the project. The aims, methods and results examine the association of Pb and Cd exposure with nasal carriage of MRSA, whilst

a. the conclusions state: "Results from our analysis of the association between Pb and both MRSA and MSSA suggest that current population exposure to Pb is associated with antibiotic resistance". There is no examination of association between detected blood Pb or Cd concentrations and the relative proportions of MRSA/MRSA carriage.

b. the abstract contains one recommendation, i.e. "additional research to highlight heavy metal exposures as a source of antibiotic resistance is needed". This does not follow from the data as the study does not investigate of differences in the proportion of S. aureus that was resistant or non-susceptible to meticillin (plus oxacillin etc, depending on the case definition for MRSA). By contrast, the first paragraph of the discussion section is based on the collected data, and had sufficient information to form conclusions and recommendations.

Response: Thank you for this thoughtful comment Language in the conclusion and abstract has been changed to be more consistent with the analyses done in this study. The conclusion of the abstract was revised as follows “Both MRSA and MSSA results suggest that general population levels of blood Pb but not Cd are associated with differences in nasal carriage of S. aureus. While further research is needed, reduction in heavy metal exposures such as lead, concurrently
with maintaining a healthy microbiota may be two modifiable options to consider in the fight against antibiotic-resistance.”

R2 Comment: 2. Certain conclusions in the results/discussion section require additional (minor) statistical analysis. For example, rather than stating that there is a trend (i.e. a "dose response" on line 223), there should be statistical test for trend, e.g. between the quartiles in tables 4 and 5.

Response: P for trend has been added to tables and results section.

R2 Comment: 3. Some interpretation of statistical data is not accurate. For example, statistical tests should be used to assess whether an association is significant, rather than "the confidence level is no longer significant" (line 223).

Response: Given the problems associated with using statistical tests with a p-value of 0.05 as a cut-off for significance, it is common practice in epidemiology to consider a 95% CI that does not cross 1.00 to be significant. To clarify this point in the text, a sentence was added to the statistical analysis section of the methods.

R2 Comment: 4. The level of technical detail varies between sections. The methods and first paragraph of the discussion section contain tight scientific text, whilst other text is less technical. For example,

a. in the abstract:
   
   i. it is not obvious that both MRSA carriage data and Pb and Cd data were available from the 'National Health and Nutritional Examination Survey', rather than an unspecified data source.

   ii. the sentence regarding 'blood Cd level' being 'significantly protective' lacks quantitative data for the reader to judge for themselves.

   iii. Is Q4 the highest (i.e. p76-p100)?

b. In table 3, the term 'veggies' could be replaced, considering this journal's international readership.

Response: The methods section of the abstract was reworded to make it clear that all data used for the analysis came from the same source. The word protective has been removed from the abstract, and the sentences have been reworded. Q (Quartile, as listed in the table abbreviations)
4, in Table 4, is by definition the highest level, including percentiles 76-100. Q4 does not appear in the abstract. The word "veggies" has been replaced with "vegetables".

R2 Comment: 5. Why was there no investigation of clinical infections with MRSA or MSSA, which are clearly the more important public health problem - if we assume that people captured in this database with nasal carriage are at higher risk of subsequent infection. On line 315, such investigation is recommended for future studies, but the limitations section does not state why such analysis was not included in this study, e.g. which data were (or were not!) available.

Response: A sentence about lack of clinical data was added to the methods and limitations.

R2 Comment: 6. There could be clearer statement in the background regarding the reasons why it is simply bad to have too much Pb or Cd in the blood, other than an indirect link to carriage (rather than infection) with S. aureus. The discussion section could repeat this briefly.

Response: A sentence has been added to the introduction to highlight some of the other adverse effects of lead and cadmium exposure.

R2 Comment: 1. Tables 1-3 can be amalgamated.

Response: Tables 1-3 have been amalgamated.

R2 Comment: 2. It is fine that the data are from 2001-2004, but there should be a brief description of the other available data sources for 2014-2017, and perhaps, briefly, why they are insufficient for such a study.

Response: Several sentences were added to the discussion section to address this.

R2 Comment: 3. Why is Race tabulated? It is unclear what the relevance is to the study. The background section does not state that the authors postulate any genetic basis a different propensity for S. aureus carriage - especially noting that the groupings are extremely non-specific, in terms of their geo-evolutionary history e.g. "white", "black", as compared to "Mexican". If these US data are a proxy for socioeconomic status, then surely other proxies for this can be added, in addition to education and income, e.g. State, etc. Relevantly, it appears that Race did not make it into the adjusted models, presumably due to insufficient evidence for association with nasal carriage of S. aureus.
Response: Race is tabulated in table 1 because it has previously been associated with both Pb exposure and MRSA colonization. Upon further examination of the models, race was added to the Pb models because it was significantly associated with Pb, MRSA, and MSSA in univariate models, and remained significant in the fully adjusted models.

R2 Comment: 4. The text in the background section regarding human immunity is interesting and generally important, but not directly related to the methods section, results section or the interpretation of the results in the conclusion (e.g. Th1 vs Th2 immune responses).

Response: This information was added to help explain the link between metal exposure and infection in general, which is part of the potential mechanism of action between Pb exposure and MRSA. Thus we believe it is important information to include, and have decided to include it in the resubmission.

R2 Comment: 5. The conclusions regarding resistance of S. aureus to the toxic effects of Cd does not consider the intermediate factors relating to plasma concentrations of these metals and carriage, e.g. immunomodulation.

Response: This conclusion has been altered to include immunomodulation.

All authors have made significant contributions to the study, and have seen and approved of the final resubmission manuscript. This manuscript has not been previously published, and is not under consideration for publication at any other journal. All authors have no conflicts of interest to disclose, and have published no closely related papers.

Thank you for your further consideration of this manuscript. We look forward to your response to these revisions.

Sincerely,

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