PEER REVIEW HISTORY

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ARTICLE DETAILS

TITLE (PROVISIONAL)	Study protocol: Characterizing the clinical, epidemiological, and etiological aspects of leptospirosis in Sri Lanka
AUTHORS	Agampodi, Suneth; Warnasekara, Janith; Jayasundara, Dinesha; Senawirathna, Indika; Gamage, Chandika; Kularatne, Senanayake; Siribaddana, Sisira; Maththias, Michael; Vinetz, Joseph

VERSION 1 – REVIEW

REVIEWER	Rasana W Sermswan Khon Kaen University, Thailand
REVIEW RETURNED	18-Jan-2019

GENERAL COMMENTS	About the study design: One of the objectives of this study is to characterize the molecular aspects of leptospirosis in Sri Lanka. The authors stated the whole genome and MLST will be applied only to new isolates. Please clarify in more details on the molecular aspects and purpose of typing in this project. This will help evaluate if the study design could completely answer your research question. More clarification is needed for the following points. 1 For confirmation of Leptospires infection, the authors mentioned MAT, culture, and qPCR. In a procedure, a rapid test was mentioned. It is not clear if the rapid test was included for confirmation or just screening. If so, pair-serum should be included to confirm the test. 2 The sample collection and procedure used for sample collection should be re-write and organize to clarify the steps for sample collection. What will be done with 2 ml blood and urine collecting during follow up? 3 What is the purpose of preserving blood sample at -80oC? 4 How data of the whole project securely kept, add-up the lab results, update, verify and shared among centers and sites in the study? 5 Where the would genome sequence and analysis will be performed? 6 Please add a reference for the PCR method for speciation as mentioned in page 15, lines 3-4. 7 Fig 1, please clarify colors of sites. For example, 2 main sites should have similar color and size. Limitation of the study Limitation on the culture of Leptospires from blood should be noted. Leptospires grow very slowly in culture and recovery rate is low. The best way to culture this spirochete from blood was

	thoroughly tested and published (Wuthiekanan et al JOURNAL OF CLINICAL MICROBIOLOGY, Apr. 2007, p. 1363–1365). Please follow her protocol to get the best out of your study. Routine blood culture and different systems have been reported to give a different outcome. Only in the first 7-10 days of symptoms that PCR or culture from blood can be positive. The author should include this point into the limitation. English usage needs approval. Also, there are some errors that need to be fixed as the list below. -Two ml of blood…. on page 12, line 5 -5ml of venous blood… on page 12, line 22 -"1300rcf" on page 12, line 24 - "foetal bovine serum" on page 14, line 4 Abbreviations that not commonly known need to be written in full name. STNPCR on page 7, line 16. MBBS graduates on page 11, line 6.

REVIEWER	Paul Le Turnier University Hospital of Nantes, Nantes, France
REVIEW RETURNED	04-Feb-2019

GENERAL COMMENTS	For authors: This is an interesting and relevant study in the context of Sri Lanka, a highly endemic area that has geographical particularities. The study protocol is clear, well designed, and supported by adapted methods. The methodology is correctly explained throughout the manuscript. The study could lead to significant new insights in several fields in leptospirosis: the identification of new Leptospira species notably. The impact of Leptospira species on clinical presentation and clinical outcome, two debated aspects will be investigated here. It could be interesting to try to distinguish leptospirosis among other undifferentiated febrile illnesses as the authors seem to plan to do. However to do it they should reconsider a little the exclusion criteria possibly to only exclude patients with diagnosis on admission (see in the comments below). Minor comments Comments refer to the single spaced journal line numbering and not from the author. 1. L 20-22, sentence could be rephrased for "No prospective has been made in Sri Lanka". Esteves et al. published in scientific Reports in 2018 a prospective study with strain typing but without description of clinical features. 2. The following exclusion criteria should be reconsidered because reject the patients with probable or suspected of meningitis or lobar pneumonia appears to me as criticable because leptospirosis can present as meningitis and pneumonia with different clinical and radiological paterns. 3. L33 typo mistake. 4. L51 56 P9 « there are probably". This phrase doesn't add anything here. 5. Please define OPD L36 P12 6. P12 L18 The similarity of infective strain in the same geographical and occupational context has been estimated to 70% bu the authors. Do the authors have a reference to help them with this evaluation? If it is overestimated it could lead to an unsufficient

	number of inclusions for the planned analysis. However it seems difficult to anticipate this point. Nevertheless, the targeted inclusions seem feasible in the sri lankan context considering the high incidence. More over although the strains are more different than anticipated, the high number of patients with confirmed leptospirosis included will be very high and allow relevant comparison and analyses for other endpoints (epidemiology, predictive score, outcomes…). 7. P17 L34 Do the authors mean “predictors of leptospirosis species” or “predictors of having a diagnosis of leptospirosis” If an analysis of the factors predicting leptospirosis is planned it is necessary to define more clearly the control group and redefine the exclusion criteria for patients with other diagnosis (differential). Is the objective of the authors to compare confirmed leptospiroses with suspected leptospirosis by first removing dengue fever, malaria, etc... cf Sukmark et al PNTD 2018 study? Or do the authors want to evaluate the predictors of leptospirosis on admission for all patients with undifferentiated febrile illnesses? If so they should consider to exclude only patients having a known differential diagnosis on admission (see Rajapakse et al PLOS NTD 2016). Then the authors should collect the biological elements used in the score of Rajapakse et al. adding the dosage of C reactive protein (which seems highly efficient to discriminate leptospirosis versus dengue, unpublished data) to further construct a relevant score or confirm the utility of the score of Rajapakse. 8. Some rapid diagnostic tests have been recently studied and could be relevant options to detect leptospirosis especially in resource limited areas. Do the authors plan to investigate this aspect regarding the high numbers of sera and urine samples they are going to collect? If not it should be considered at least to evaluate the sensitivity of those RDTs (sera or urine). To address the diagnosis capacity it would be necessary to constitute an homogeneous negative control group with systematic and controlled diagnostic analysis for differential diagnoses. 9. Follow up medical visit is set at 2 or 3 weeks after initial admission and inclusion? (cf P12 L 11 and P13 L20) 10. L45-47 “using CDC…” is redundant with the first sentence of the paragraph. 11. it is understandable to set the follow up visit 2 weeks after the inclusion (admission) for risk of follow-up loosing and for doing the PCR test in urine. However, it could be too soon to detect a significant positive MAT and especially to assess the true presumptive infecting serogroup. Indeed coagglutinins are frequent in early samples. A delayed sample at least over 1 month should be considered to really ascertain the infecting serovar/serogroup and compare it with the sequencing of the strain. The cut off set at 1/800 is up to date and strongly specific but once again it could miss some patients. Authors could consider a slightly diminished titer threshold (1/400). Seroconversion from negative to positive suggests positive with at least MAT of 100? 12. P15 L18 do you mean “we will use” L 20 do you mean “allow”? 13. The authors could clarify on how they will concretely identify a new strain. Are they defined by the strains found in the patients with positive 16S RNA or LipL32 genes PCR that will not be detected by species-specific primers? If so it should added somewhere in the methods 14. P17 L 54 Do you mean “on a continuous basis”?

REVIEWER	Anou Dreyfus Swiss Tropical and Public Health Institute, Basel, Switzerland
REVIEW RETURNED	06-May-2019

GENERAL COMMENTS	General comment In my opinion, the proposed study is highly useful, locally and internationally and will tackle many open questions, filling important knowledge gaps. The study is built on a good basis from previous work done in Sri Lanka and on current state of knowledge. While I enjoyed reading certain parts, such as the introduction, other parts in the methodological section seem unstructured with a lot of repetition (i.e. Procedure). These could still be improved. While the SOPs of the diagnostics are thoroughly described, I find the description of the collection of epidemiological data too scarce. I could not find a paragraph, which describes, what data is exactly collected in the interviews. Are you going to ask about work, residential and leisure exposure (working in rice paddy fields, contact to cattle, dogs, rodents etc.)? If you already conduct interviews, why not ask and try to find risk factors for leptospirosis, which are not only based on genetical analysis but epidemiological data? Also you could elaborate a bit more precisely on the term “microgeography”. How will you merge the data, collected in different hospitals into one database? You could elaborate a bit more on data collection and management. The forms I have to fill in to submit a study proposal, always contain a section, where the study management, the exact timeline, the team who will implement the study is described. This seems missing, so the feasibility in these terms cannot be evaluated. Also, I miss the section which describes the impact of the study. I.e. that the study will contribute to the improvement of patient management etc.. Are you going to validate test protocols useful for different settings? I miss the translation from academic knowledge to tangible outputs for doctors, patients and society (apart from communicating test results in a timely manner). Language: certain parts of the draft still need improvement of the English. Often the nouns are not preceded by the definite article (“the”). Some sentences are unclear. In some tables, Leptospira is not italic. In detail: Title: While it is correct that knowledge is accrued to contribute to the assessment of the disease burden in Sri Lanka, I find the term “global disease burden assessment” in the title a bit exaggerated, as the data is collected in one country and I doubt that the study is longitudinal and representative enough to collect incidence data, which is inevitable for such an assessment. I would focus in the title on what is done, i.e. the undifferentiated fever study in Sri Lanka. Page 3, line 12: I would add longitudinal/prospective in front of “study”

	Page 3, line 44: what do you mean with “newly isolated”? Page 4, line 6: “reasonable request” may not be concrete enough Page 4, line 13: I would add “at the time of publication of this protocol” Page 4, line 22: has not been carried out would be the correct tense (English). These kind of grammar mistakes occur throughout the protocol, please revise. Page 4, line 27: Leptospira italic…..(please revise everywhere where not italic) Page 6, line 24: diagnostic Page 6, line 44-54: please revise sentence Page 6, line 54: need not needs (now I will stop with language correction) Page 7, line 15: Please define microgeography Page 7, line 36: is still unknown in Sri Lanka Page 9: why not add an objective on finding occupational, residential and leisure risk factors for leptospirosis? Knowing epidemiological risk factors can also guide clinicians in a risk assessment and diagnosis. Since you conduct the interviews anyway? Page 10, Inclusion criteria need revision: how is the fever measured? Self reported fever: it always should be measured by a clinician. Page 10 and 11, Exclusion criteria: Influenza-like-illness is very typical for leptospirosis and should not be an exclusion criteria. Meningitis and CNS symptoms can be caused by Leptospira and should not be an exclusion criteria. Are you sure you can differentiate lower respiratory tract infection from pulmonary hemorrhage in all your hospital settings (I am not a clinician)? What if antibiotics have already been given? Page 11, line 13: did you ever write the full word for MBBS? Page 11, line 27: here I would expect a few lines on consent and confidentiality Page 11, line 29: the whole paragraph on Procedure does not seem very structured and is a bit difficult reading…Also further down many points are repeated. I would invest a little bit more time in it. Sometimes a figure can be nice…. Please elaborate a bit more on the data you will collect. On what will patients be interviewed? What will the questionnaire include? Why not have the questionnaire as an annex to this protocol? Your SOPs on diagnostics are very detailed, but the collection of clinical and epidemiological data is kept very short. Page 12, line 11: why will you collect only 2 ml? Why not collect more and have a good biobank for follow-up questions? In general, as long as the patient can tolerate it and it is ethically accepted, I would collect higher amounts of blood for a biobank (first and second sample). Then you can compare new diagnostic tests with your results and validate new methods for the future. I would also recommend writing the consent forms in a manner that other pathogens (i.e. Rickettsia, Q-fever, Influenza, Dengue etc) and other follow-up questions can be tested in the future. It would be nice to know the proportion of leptospirosis in comparison with other febrile illnesses.

	Page 13, line 15: this last sentence seems a bit out of context Page 13, line 24: I would start with case definitions: what is a probable case, what is a confirmed case…Tables look good… Page 13, lines 47: “at the next step”….please revise the whole sentence Page 13, line 52: from negative to positive..until which titre is a patient negative? ≤1: 100? Is the titre for a positive case ≥1:100? How do you interpret seroconversion if antibiotics have already been administered? Page 14, line 15: motile, not mortile Page14, line 22. Please revise sentence. Page 14, line 34? Is it useful to extract DNA from serum? Page 14: Diagnosis: what about the rapid diagnostic tests kits you menation at the end in the ethics paragraph? Page 15, line 20: English no good ;) Page17, line 10: you describe the data management, but what the actual data consists of is somehow missing…

VERSION 1 – AUTHOR RESPONSE

Reviewer 1

Reviewer Comment	Author response
For confirmation of Leptospires infection, the authors mentioned MAT, culture, and qPCR. In a procedure, a rapid test was mentioned. It is not clear if the rapid test was included for confirmation or just screening. If so, pair-serum should be included to confirm the test.	Rapid diagnostic kits were used to provide onsite feedback to treating physicians. It was not a part of the main study.
The sample collection and procedure used for sample collection should be re-write and organize to clarify the steps for sample collection. What will be done with 2 ml blood and urine collecting during follow up?	According to the reviewer comment the methodology section was rewritten. The blood will be used for paired sera and the DNA extracted from the urine for PCR analysis to investigate long term urinary sheding of leptospira. Page 11 Line 22-26 Page 12 Line 1-12
What is the purpose of preserving blood sample at -80^{o}C?	Aliquots will be prepared from whole blood and serum. They will be stored in -80^{o}C for future biochemical, serological and PCR assays, specially to validate the new diagnostic tests. Page 12 Line 2
How data of the whole project securely kept, add-up the lab results, update, verify and shared among centers and sites in the study	This has been updated and revised in data management and analysis section. The password is given to every investigator. Data base will be updated regularly. Page 17 Line 9-12
Where the whole genome sequence and analysis will be performed	MinION Nanopore sequencing will be done using nanopore device in Leptospirosis research lab, Faculty of Medicine and Allied Sciences . PacBio third generation

	sequencing will be done at Institute for Genome medicine, University of California, San Diego. Analysis will be done at Leptospirosis research laboratory Faculty of Medicine and Allied Sciences, RUSL This is also mention in the molecular studies part of the methodology section. Page 15 Line 12-14
Please add a reference for the PCR method for speciation as mentioned in page 15, lines 3-4	Thanks. The particular reference was added Page 15 Line 6
Fig 1, please clarify colors of sites. For example, 2 main sites should have similar color and size	We have revised the figure 1, considering the editorial comments as well.
Limitation on the culture of Leptospires from blood should be noted. Leptospires grow very slowly in culture and recovery rate is low. The best way to culture this spirochete from blood was thoroughly tested and published (Wuthiekanan et al JOURNAL OF CLINICAL MICROBIOLOGY, Apr. 2007, p. 1363–1365). Please follow her protocol to get the best out of your study. Routine blood culture and different systems have been reported to give a different outcome. Only in the first 7-10 days of symptoms that PCR or culture from blood can be positive. The author should include this point into the limitation	Limitation section was modified according to the reviewer comment. As you have suggested we have referred this article and adopted the best way which is feasible for our study. Page 13 line 16 Page 3 Line 15- 21
English usage needs approval. Also, there are some errors that need to be fixed as the list below. Two ml of blood…. on page 12, line 5 5ml of venous blood… on page 12, line 22 "1300rcf" on page 12, line 24 "foetal bovine serum" on page 14, line 4	All these minor comments were addressed Page 11 Lines 24-26 and page 12 Lines 1-12

Reviewer 02

Reviewer comment	Author response
L 20-22, sentence could be rephrased for "No prospective has been made in Sri Lanka". Esteves et al. published in scientific Reports in 2018 a prospective study with strain typing but without description of clinical features.	This was corrected according to the comment Page 7 Line 8-9
The following exclusion criteria should be reconsidered because reject the patients with probable or suspected of meningitis or lobar pneumonia appears to me as criticable because leptospirosis can present as meningitis and pneumonia with different clinical and radiological paterns.	We fully agree with this comment. As the study progresses we also understood leptospirosis patients present with lower respiratory tract infection and meningitis like pictures. So the exclusion criteria were modified Page 9 lines 24-26
L33 typo mistake	Corrected
L51 56 P9 « there are probably". This phrase doesn't add anything here	This was corrected Page 8 Line 22
5.Please define OPD L36 P12	Term OPD was define according to the comment Page 10 Line 15
6. P12 L18 The similarity of infective strain in the same geographical and occupational context has been estimated	We had the same dilemma as reviewers due to lack of data for sample size

to 70% by the authors. Do the authors have a reference to help them with this evaluation? If it is overestimated it could lead to an insufficient number of inclusions for the planned analysis. However it seems difficult to anticipate this point. However it seems difficult to anticipate this point. Nevertheless, the targeted inclusions seem feasible in the sri lankan context considering the high incidence. More over although the strains are more different than anticipated, the high number of patients with confirmed leptospirosis included will be very high and allow relevant comparison and analyses for other endpoints (epidemiology, predictive score, outcomes…)	calculation. We do not have a reference for the claim and that's why we used "assuming". We slightly changed the sentence in the revised manuscript. Page 11 Line 22
7. P17 L34 Do the authors mean "predictors of leptospirosis species" or "predictors of having a diagnosis of leptospirosis". If an analysis of the factors predicting leptospirosis is planned it is necessary to define more clearly the control group and redefine the exclusion criteria for patients with other diagnosis (differential). Is the objective of the authors to compare confirmed leptospiroses with suspected leptospirosis by first removing dengue fever, malaria, etc... cf Sukmark et al PNTD 2018 study? Or do the authors want to evaluate the predictors of leptospirosis on admission for all patients with undifferentiated febrile illnesses? If so they should consider to exclude only patients having a known differential diagnosis on admission (see Rajapakse et al PLOS NTD 2016). Then the authors should collect the biological elements used in the score of Rajapakse et al. adding the dosage of C reactive protein (which seems highly efficient to discriminate leptospirosis versus dengue, unpublished data) to further construct a relevant score or confirm the utility of the score of Rajapakse.	We were planning to look at the predictors of leptospirosis and predictors of severe disease. According to the Rajapaksa et al paper, CRP seems to be a strong predictor of the diagnosis of leptospirosis. However, in the usual settings, most of the clinicians are not requesting CRP unless the patient is having moderate to severe complications. We have not specifically included CRP in the list of biochemical tests for this reason. Rather than predicting leptospirosis, we will be predicting the severe disease among confirmed cases, as those cases will be having more biochemical parameters available. We revised the manuscript accordingly. Nevertheless, we might be able to do the suggested analysis based on the availability of investigation results. Page 17 line 15
8. Some rapid diagnostic tests have been recently studied and could be relevant options to detect leptospirosis especially in resource limited areas. Do the authors plan to investigate this aspect regarding the high numbers of sera and urine samples they are going to collect? If not it should be considered at least to evaluate the sensitivity of those RDTs (sera or urine). To address the diagnosis capacity it would be necessary to constitute an homogeneous negative control group with systematic and controlled diagnostic analysis for differential diagnoses.	Thank you for your valuable comment. Yes, one of the idea of having a biobank is testing newly available rapid diagnostic kits. We are planning to implement this in due course. We are planning to arrive at a diagnosis for all "negative" cases as you suggested to have a well defined control group.
9. Follow up medical visit is set at 2 or 3 weeks after initial admission and inclusion? (cf P12 L 11 and P13 L20)	Both places were corrected accordingly by adding 3 weeks Page 10 Line 3 and Page 12 line 10
L45-47 "using CDC…" is redundant with the first sentence of the paragraph.	This suggestion was included Page 13 line 8
it is understandable to set the follow up visit 2 weeks after the inclusion (admission) for risk of follow-up loosing and for doing the PCR test in urine. However, it could be too soon to detect a significant positive MAT and especially to assess	We extended the follow up period to three weeks after onset of disease. Extending the time period beyond that would not be

the true presumptive infecting serogroup. Indeed, coagglutinins are frequent in early samples. A delayed sample at least over 1 month should be considered to really ascertain the infecting serovar/serogroup and compare it with the sequencing of the strain.	feasible as the patients might not turn-up after they recover from the illness Page 14 line 5, Page 11 line 3, page 12 line 10,
The cut off set at 1/800 is up to date and strongly specific but once again it could miss some patients. Authors could consider a slightly diminished titer threshold (1/400).	We have changed MAT cutoff titre to 1/400 as you suggested (and this was the practice we had up to now for reports) Page 14 line 2
Seroconversion from negative to positive suggests positive with at least MAT of 100?	Yes. As suggested seroconversion titre was changed. Page 14 line 1
P15 L18 do you mean “we will use” L 20 do you mean “allow”?	Yes. Thanks for pointing this out. This sentence was rewritten according to the comment Page 15 lines 3-6
The authors could clarify on how they will concretely identify a new strain. Are they defined by the strains found in the patients with positive 16S RNA or LipL32 genes PCR that will not be detected by species-specific primers? If so it should added somewhere in the methods	Yes, we agree with your comment. New strain identification will be done only for the positive cultures using whole genome sequencing. For species level identification (before WGS) we will use previously validated qPCR protocol. Page 15 lines 3-6
P17 L 54 Do you mean “on a continuous basis”?	Yes, corrected Page 17 line 25

Reviewer 03

Reviewer comment	Author response
General comments: In my opinion, the proposed study is highly useful, locally and internationally and will tackle many open questions, filling important knowledge gaps. The study is built on a good basis from previous work done in Sri Lanka and on current state of knowledge. While I enjoyed reading certain parts, such as the introduction, other parts in the methodological section seem unstructured with a lot of repetition (i.e. Procedure). These could still be improved. While the SOPs of the diagnostics are thoroughly described, I find the description of the collection of epidemiological data too scarce. I could not find a paragraph, which describes, what data is exactly collected in the interviews. Are you going to ask about work, residential and leisure exposure (working in rice paddy fields, contact to cattle, dogs, rodents etc.)? If you already conduct interviews, why not ask and try to find risk factors for leptospirosis, which are not only based on genetical analysis but epidemiological data? Also you could elaborate a bit more precisely on the term “microgeography”. How will you merge the data, collected in different hospitals into one database? You could elaborate a bit more on data collection and management. The forms I have to fill in to submit a study	We have added a new section on variables and epidemiological data. Page 10 line 21- page 11 line 8 In addition all the comments were addressed as detailed under the specific comments.

proposal, always contain a section, where the study management, the exact timeline, the team who will implement the study is described. This seems missing, so the feasibility in these terms cannot be evaluated. Also, I miss the section which describes the impact of the study. I.e. that the study will contribute to the improvement of patient management etc.. Are you going to validate test protocols useful for different settings? I miss the translation from academic knowledge to tangible outputs for doctors, patients and society (apart from communicating test results in a timely manner) Language: certain parts of the draft still need improvement of the English. Often the nouns are not preceded by the definite article ("the"). Some sentences are unclear. In some tables, Leptospira is not italic	
Title: While it is correct that knowledge is accrued to contribute to the assessment of the disease burden in Sri Lanka, I find the term "global disease burden assessment" in the title a bit exaggerated, as the data is collected in one country and I doubt that the study is longitudinal and representative enough to collect incidence data, which is inevitable for such an assessment. I would focus in the title on what is done, i.e. the undifferentiated fever study in Sri Lanka.	We full agree on this comment. The issue we had was that the NIH grant included this phrase. However, we have removed the "global disease burden assessment" from the revised tile.
Page 3, line 12: I would add longitudinal/prospective in front of "study"	This was corrected as per reviewer comment Page 2 line 12
Page 3, line 44: what do you mean with "newly isolated"?	This mean all isolate of Leptospira from the patient samples Page 2 line 20-21
Page 4, line 6: "reasonable request" may not be concrete enough	This sentence is revised. Page 3 line 2-5
Page 4, line 13: I would add "at the time of publication of this protocol"	The given term was added Page 3 line 7
Page 4, line 22: has not been carried out would be the correct tense (English). These kind of grammar mistakes occur throughout the protocol, please revise.	Thank you for your comment we have revised those mistakes Page 3 Line 10
Page 4, line 27: Leptospira italic…..(please revise everywhere where not italic)	This error was corrected in the whole document
Page 6, line 24: diagnostic	Corrected Page 5 line 11
Page 6, line 44-54: please revise sentence	Corrected
Page 6, line 54: need not needs (now I will stop with language correction)	This was corrected Page 5 line 24
Page 7, line 15: Please define microgeography	Corrected as "geographical"
Page 7, line 36: is still unknown in Sri Lanka	In Sri Lanka is added Page 6 line 16
Page 9: why not add an objective on finding occupational, residential and leisure risk factors for leptospirosis? Knowing	We can actually do this using the available data. However, what we publish here is the

epidemiological risk factors can also guide clinicians in a risk assessment and diagnosis. Since you conduct the interviews anyway?	grant proposal, so that we avoided adding new objectives here.
Page 10, Inclusion criteria need revision: how is the fever measured? Self reported fever: it always should be measured by a clinician	Fever will be measured by digital thermometer Self-reported fever is included as some patients may not have fever at the time of admission(Due to antibiotics, antipyretics or self recovery from fever) but still it could be leptospirosis)
Page 10 and 11, Exclusion criteria: Influenza-like-illness is very typical for leptospirosis and should not be an exclusion criteria. Meningitis and CNS symptoms can be caused by Leptospira and should not be an exclusion criteria. Are you sure you can differentiate lower respiratory tract infection from pulmonary hemorrhage in all your hospital settings (I am not a clinician)? What if antibiotics have already been given?	We agree with your comment. As the study progresses we also understood leptospirosis patients present with lower respiratory tract infection and meningitis like pictures. So the exclusion criteria was modified Page 9 lines 24 -26
Page 11, line 13: did you ever write the full word for MBBS?	The term MBBS was elaborated Page 10 line 4
Page 11, line 27: here I would expect a few lines on consent and confidentiality	We had a separate section on ethics. However we added few sentences here in the revised manuscript. Page 10 line 22
Page 11, line 29: the whole paragraph on Procedure does not seem very structured and is a bit difficult reading…Also further down many points are repeated. I would invest a little bit more time in it. Sometimes a figure can be nice… Please elaborate a bit more on the data you will collect. On what will patients be interviewed? What will the questionnaire include? Why not have the questionnaire as an annex to this protocol? Your SOPs on diagnostics are very detailed, but the collection of clinical and epidemiological data is kept very short.	We have added a paragraph on the variables and the questions.
Page 12, line 11: why will you collect only 2 ml? Why not collect more and have a good biobank for follow-up questions? In general, as long as the patient can tolerate it and it is ethically accepted, I would collect higher amounts of blood for a biobank (first and second sample). Then you can compare new diagnostic tests with your results and validate new methods for the future. I would also recommend writing the consent forms in a manner that other pathogens (i.e. Rickettsia, Q-fever, Influenza, Dengue etc) and other follow-up questions can be tested in the future. It would be nice to know the proportion of leptospirosis in comparison with other febrile illnesses	We all agree with this comment! This was suggested by the ERC and we had no option but to comply.
Page 13, line 15: this last sentence seems a bit out of context	This was corrected

	Page 12 lines 7-10
Page 13, line 24: I would start with case definitions: what is a probable case, what is a confirmed case…Tables look good…	This was added at the end of page 12
Page 13, lines 47: “at the next step”….please revise the whole sentence	Whole sentence was rewritten Page 13 lines 9-10
Page 13, line 52: from negative to positive..until which titre is a patient negative? ≤1: 100? Is the titre for a positive case ≥1:100? How do you interpret seroconversion if antibiotics have already been administered?	Cut of titre will be set as 1/400 for acute samples Page 14 line2 Seroconversion titre will be set at 1/100 So it is unlikely to miss patients whose antibody production is hindered by antibiotics Usually patients are admitted around 3-5 days of onset of fever by which time immune reaction is likely to be triggered Page 14 line 1
Page14, line 22. Please revise sentence.	This sentence was revised Page 14 lines 13-15
Page 14, line 34? Is it useful to extract DNA from serum?	Yes, we used serum to compare the ct value of q PCR assay with whole blood. Because there was previous publication indicating low Ct values in serum compared to whole blood Agampodi SB, Matthias MA, Moreno AC, Vinetz JM. Utility of Quantitative Polymerase Chain Reaction in Leptospirosis Diagnosis: Association of Level of Leptospiremia and Clinical Manifestations in Sri Lanka. Clin Infect Dis [Internet]. 2012 May 1;54(9):1249–55. Available from: https://academic.oup.com/cid/article-lookup/doi/10.1093/cid/cis035
Page 14: Diagnosis: what about the rapid diagnostic tests kits you mention at the end in the ethics paragraph?.	Rapid diagnostic kits were often requested by the clinicians as bed site tests. As establishment of the laboratory takes time, at the beginning we will be offering

	commercially rapid diagnostic tests as a service, not as a research component.
Page 15, line 20: English no good ;)	This whole section was revised Page 15 lines 3-6
Page17, line 10: you describe the data management, but what the actual data consists of is somehow missing	Missing data management is added to the revised manuscript

VERSION 2 – REVIEW

REVIEWER	Rasana W Sermswan Department of Biochemistry, Faculty Medicine, Khon Kaen University, Khon Kaen, Thailand.
REVIEW RETURNED	11-Jun-2019

GENERAL COMMENTS	-The manuscript has no page No. that difficult to check according to their response. -The abstract is too long that can make more concise. I'm not sure about format though but list of objectives in the introduction is quite strange. -The 5th objective should be re-write. -The procedure mentioned about blood and urine collection without describing the purpose while blood and urine that wrote in the sample collection during follow-up has more details. Will their purpose the same? To avoid confusion, would it be better to describe them all in the procedure? -Thanks for adding 3 references for PCR detection in urine, however, which one will be follow? If you're going to modify or try condition, better write the optimised one. Other people then can easily follow. Some errors were still detected as track change in the file. - The reviewer also provided a marked copy with additional comments. Please contact the publisher for full details.

REVIEWER	Paul Le Turnier, MD Infectious diseases department, Nantes University Hospital, Nantes, France
REVIEW RETURNED	12-Jun-2019

GENERAL COMMENTS	As mentioned in the previous review, this study could provide interesting information on leptospirosis in Sri Lanka, an area of high endemicity, but also serve as a model for studies in other areas where leptospirosis remains poorly studied. The remarks made during the previous review have been taken into account and the protocol has become more precise and relevant. I think it is worth publishing provided that the level of English is further improved, especially in the modified parts compared to the original manuscript.

REVIEWER	Anou Dreyfus Please check in the former review of the same paper
REVIEW RETURNED	24-Jun-2019

GENERAL COMMENTS	I would write: "seroconversion from negative to positive (<1:100 to ≥1:100); 4-fold increase in titer between acute-phase and convalescent-phase (follow-up) samples; or a single titer of ≥400".

VERSION 2 – AUTHOR RESPONSE

Reviewer: 1

Reviewer Name: Rasana W Sermswan

Institution and Country: Department of Biochemistry, Faculty Medicine, Khon Kaen University, Khon Kaen, Thailand.

-The manuscript has no page No. that difficult to check according to their response.

Page numbers are included in the revised manuscript

-The abstract is too long that can make more concise.

Abstract is slightly shortened

I'm not sure about format though but list of objectives in the introduction is quite strange.

-The 5th objective should be re-write.

The objectives were the once submitted to NIH as the grant proposal and these are to be keep as it is because, this is how it appears in the grant proposal (approved)

-The procedure mentioned about blood and urine collection without describing the purpose while blood and urine that wrote in the sample collection during follow-up has more details. Will their purpose the same? To avoid confusion, would it be better to describe them all in the procedure?

The revised manuscript has given in text reference to sample collection details and moved small description from the general description of the procedure.

-Thanks for adding 3 references for PCR detection in urine, however, which one will be follow? If you're going to modify or try condition, better write the optimised one. Other people then can easily follow.

Since these optimization were not done yet. We cant say what we will be using. However, we have added a line saying that we will optimize the procedure.

Some errors were still detected as track change in the file.

All track changes are removed now.

Reviewer: 2

Reviewer Name: Paul Le Turnier, MD

Institution and Country: Infectious diseases department, Nantes University Hospital, Nantes, France

As mentioned in the previous review, this study could provide interesting information on leptospirosis in Sri Lanka, an area of high endemicity, but also serve as a model for studies in other areas where leptospirosis remains poorly studied. The remarks made during the previous review have been taken into account and the protocol has become more precise and relevant. I think it is worth publishing provided that the level of English is further improved, especially in the modified parts compared to the original manuscript.

Thanks

Reviewer: 3

Reviewer Name: Anou Dreyfus

Institution and Country: Please check in the former review of the same paper

Please leave your comments for the authors below

I would write: "seroconversion from negative to positive (<1:100 to ≥1:100); 4-fold increase in titer between acute-phase and convalescent-phase (follow-up) samples; or a single titer of ≥400".

Done with a small change