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Epidemiologie, Diagnostik und Therapie von Tuberkulose und anderen Infektionskrankheiten in Sub-Sahara Afrika

vorgelegt von
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Diese Arbeit ist meiner Frau Irene und meinen Söhnen Maximilian und Clemens gewidmet.

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1. Zusammenfassung der Habilitationsarbeit

Epidemiologie, Diagnostik und Therapie von Tuberkulose und anderen Infektionskrankheiten in Sub-Sahara Afrika

Einleitung

In der vorliegenden Habilitationsarbeit werden sowohl Studien zur Verbsserung der Tuberkulosetherapie in Tansania und Südafrika, zur Verbesserung des Therapiemonitorings, sowie verschiedene Aspekte der Infektionsepidemiologie von durch Arthropoden übertragenen Erkrankungen in Tansania zusammengefasst; und ein Ausblick auf aktuell laufende Arbeiten gegeben.

Infektionskrankheiten stehen in der weltweiten Todesursachenstatistik weiterhin weit vorne; mit 8,4 Millionen Todesfällen im Jahr 2015, 15% der gesamten globalen Todesfälle im Jahr 2015 (1). Die größte Zahl an Todesfällen aller Infektionskrankheiten weltweit fordert aktuell die Tuberkulose mit 1,5 Millionen {WHO, 2020 #743}.

Vor allem in Afrika zeigt sich ein höherer Anteil der Infektionskrankheiten an der gesamten Sterblichkeit. Dieser Kontinent zeigt weltweit die höchste Inzidenz der Tuberkulose (TB); mit 390 Neuerkrankungen pro 100.000 Personen pro Jahr (global: 142 pro 100.000), und einer noch relativ hohen Sterblichkeit von 27% (global: 17%; (2).

TB beim Menschen wird von säurefesten Stäbchenbakterien aus der Gattung *Mycobacteriaceae* verursacht. Hier sind besonders die Spezies *M. tuberculosis, M. africanum und M. bovis* relevant. Die Infektion erfolgt meist über Inhalation bakterienhaltiger Tröpfchen, und Aufnahme der Bakterien in pulmonale Makrophagen. Etwa jeder zehnte Infizierte erkrankt; bei HIV-infizierten Patienten ist dieser Anteil etwa fünffach höher. Die Sterblichkeit innerhalb von zwei Jahren ohne Therapie wird in historischen Schriften mit etwa 50% angegeben (3).

Die Tuberkulose wurde im Jahr 1993 durch die WHO zum globalen Gesundheitsnotfall erklärt, nachdem die Erkrankungs – und Sterblichkeitszahlen infolge der Ausbreitung von HIV/AIDS, wodurch die Erkrankung an Tuberkulose begünstigt wird, dramatisch angestiegen waren. Bisher konnte durch globale Bemühungen zwar die Sterblichkeit an TB gesenkt werden, die Zahl der Neuerkrankungen ist jedoch noch nicht deutlich rückläufig, wodurch die Ziele der WHO, die Inzidenz bis 2030 um 80% zu senken, gefährdet sind (2).

Um eine normale, nicht-resistente TB zu heilen, wird derzeit eine Therapie mit vier Medikamenten benötigt, die über sechs Monate eingenommen werden muss. Hiermit wird in Studien eine Heilungsrate von bis zu 95 % erreicht, im globalen Mittel aktuell 75 % (2).

Bei resistenter TB erstreckt sich die Therapiedauer über bis zu 18 Monate, mit einer höheren Zahl von Medikamenten, mehr Nebenwirkungen und geringeren Aussichten auf Heilung. Diese lange Therapiedauer stellt eine erhebliche Bürde für Patienten und Gesundheitswesen dar, verursacht erhöhte Kosten, und wird im globalen Kontext nur von 52% der Patienten erfolgreich abgeschlossen (2). Kürzere, einfachere Therapien und neue Medikamente gegen resistente TB sind deshalb – neben besseren Diagnostika und der Entwicklung eines wirksamen Impfstoffs - Prioritäten in der TB-Forschungsagenda, wie von der WHO in ihrer „Strategy to End TB“ beschrieben (4) .

Neben hoher Mortalität, verursachen andere Infektionskrankheiten weiterhin hohe Erkrankungszahlen, und dadurch und durch postinfektiöse Beschwerden und Einschränkungen einen hohen Verlust an gesunden Lebensjahren. Unter den in dieser Arbeit untersuchten Erkrankungen ist hier insbesondere das Chikungunya Fieber zu nennen, dass durch monate- bis jahrelange Gelenkschmerzen nach Erkrankung oft eine länger dauernde Arbeitsunfähigkeit verursacht (5).

Das Rifttalfieber (RTF), welches wie Chikungunya von Moskitos übertragen wird, verläuft nur bei einem kleinen Anteil der Erkrankten schwerwiegend, verursacht aber auch schwere ökonomische Schäden

durch Befall von Nutztierherden. Dies hat schwere Erkrankungen, und bei trächtigen Tieren einen Abort zur Folge.

Ebenso zur Gruppe der durch Arthropoden übertragenen Erkrankungen gehören die Rickettsiosen. Diese intrazellulären Bakterien werden vor allem durch Zecken übertragen, und verursachen ein Spektrum verschiedener Erkrankungen, die meist durch Fieber, Ausschlag und Eschar (schwärzliche Läsion an der Bisstelle) charakterisiert sind. Von dieser Erregerfamilie werden sowohl ungefährliche Erkrankungen wie das afrikanische Zeckenbißfieber (*Rickettsia africae*) verursacht, als auch Erkrankunge mit hoher Mortalität wie das epidemische Fleckfieber (*Rickettsia prowazekii*). Serologisch werden die Rickettsiosen in die Zeckenbiß – Fleckfiebergrupe, (R. africae, R. conorii, R.massiliae), sowie in die epidemische Fleckfiebergruppe (*R. typhi, R. prowazekii*) eingeteilt.

Verbesserte Therapie der Tuberkulose

Im Rahmen des PanACEA Netzwerkes, dessen Ziel die klinische Erprobung neuer Medikamente gegen Tuberkulose ist, wurde der Wirkstoffkandidat Sq109 in klinischen Studien erprobt (*Heinrich et al., Early phase evaluation of SQ109 alone and in combination with rifampicin in pulmonary TB patients;* (6)). Sq109 ist ein asymmetrisches Diamin, das aus einem Screeningprogramm einer chemischen Library von Ethambutol-Derivaten der Firma Sequella, Inc., hervorgegangen ist (7). Die Substanz zeigte in vitro sowie im Mausmodell gute Wirksamkeit, die eine Überlegenheit gegenüber der Muttersubstanz Ethambutol vermuten ließ (8), und auf eine synergistische Wirkung mit dem Standardtuberkulosemedikament Rifampicin hindeute. Sq109 wirkt unter anderem auf das Tranporterprotein *mmpl3,* welches Trehalosemonomykolat, einen Bestandteil für den Zusammenbau von Mykolsäuren in der Zellwand aus dem Zytosol, dem Ort der Synthese, herauspumpt, zum Ort des Verbrauchs (9). Dieser Mechanismus unterscheidet sich von dem der Muttersubstanz, dementsprechend finden sich keine Kreuzresistenzen (7). Bemerkenswert war, dass die überlegene Wirkung von Sq109 iM Tiermodell erst nach 30 Tagen Therapie in nur geringem Maß nachweisbar war, und der Effekt sich erst später verstärkte (8).

In der Phase I- Evaluation war Sq109 von gesunden menschlichen Probanden in allen Dosierungen gut vertragen worden (7). Untersuchungen des Metabolismus von Sq109 hatten eine Metabolisierung über die hepatischen CYP450 Isoenzyme 2C19 und 2D6 gezeigt.

An der hier präsentierten Studie wirkte ich maßgeblich als Sponsor Medical Expert mit, verantwortlich für Protokolldesign, protokollkonforme Studiendurchführung, wissenschaftliche Auswertung und Publikation. Die Studie wurde an zwei Zentren in Kapstadt, Südafrika, durchgeführt und hatte die klinische Phase IIA – Erprobung von Sq109 an Patienten mit neudiagnostizierter pulmonaler Tuberkulose zum Ziel, um die Nebenwirkungsrate, Wirksamkeit von drei verschiedenen Dosierungen, und Pharmakokinetik bei einer Gabe über 14 Tage zu beschreiben. Hierzu wurde ein sechsarmiges, prospektiv randomisiertes, teilverblindetes, kontrolliertes Studiendesign mit einer Probandenzahl von 90 gewählt.

In drei Armen erhielten Patienten Sq109 als Monotherapie über 14 Tage in den Dosierungen 75mg, 150mg und 300mg täglich; in zwei Armen wurden Dosierungen von 150mg und 300mg in Kombination mit Rifampicin gegeben. Im Kontrollarm wurde Rifampicin als Monotherapie gegeben. Die Sq109-Dosis wurde verblindet; die Gabe von Rifampicin konnte nicht verblindet werden, da dieses Medikament bekanntermaßen eine orangene Färbung von Körperflüssigkeiten verursacht.

Sq109 zeigte insgesamt ein günstiges Nebenwirkungsprofil. Milde und moderate, dosisabhängige Nebenwirkungen im Verdaungstrakt zeigten sich als häufigste unerwünschte Ereignisse, es wurden keine Auswirkungen auf die kardiale Reizleitung und – Rückbildung im EKG gesehen. Ein Patient aus der 300mg – Gruppe verstarb an einer schweren Lungenblutung elf Tage nach der letzten Dosis Sq109. Dies wurde als beschriebene Komplikation der Grunderkrankung ohne ursächlichen Zusammenhang mit dem Studienmedikament angesehen.

1. Zusammenfassung der Habilitationsarbeit

Zur Abschätzung der Wirksamkeit wurde die durchschnittliche tägliche Reduktion der Bakterienlast im Sputum (log_{10}colony forming units; log_{10}CFU) gemessen. Diese betrug 0.093 log_{10}CFU/Tag (95% Konfidenzintervall (KI) 0.126–0.059) für die Rifampicin – Kontrollgruppe, 0.133 (0.166–0.100) für Rifampicin plus 150 mg SQ109, und 0.089 (0.121–0.057) für Rifampicin plus 300 mg SQ109. In den Armen mit Sq109 – Monotherapie zeigte sich keine signifikante Reduktion der Keimlast.

Die höchsten gemessenen Plasmaspiegel für Sq109 lagen unterhalb der minimalen Hemmkonzentrationen der von den Patienten isolierten Erreger, ein Phänomen, welches sich für dieses Medikament bereits in Mausstudien gezeigt hatte. Die Kombinationstherapie mit Rifampicin verursachte eine deutliche Reduktion der Sq109-Plasmaspiegel bei einer Dosis von 150mg, was mit der bereits beschriebenen Induktion der CYP450 Isoenzyme 2C19 und 2D6 durch Rifampicin erklärbar ist, aber bei der höheren Sq109 -Dosis von 300mg in nur sehr geringem Maß beobachtet wurde.

Die nicht nachweisbare Wirkung von Sq109 auf die Bakterienlast lässt mehrere mögliche Interpretationen zu. Da die Plasmaspiegel bei Mäusen und Menschen unterhalb der Hemmkonzentration lagen, war die Anreicherung des Wirkstoffs in der Lunge maßgeblich für die Wirksamkeit. Diese Anreicherung war bei Mäusen mit bis zu 40-fach über dem Plasma liegenden Konzentrationen nachgewiesen worden (7, 10). Naturgemäß ist es beim Menschen kaum möglich, Lungengewebe zu gewinnen, um einen ähnlichen Nachweis zu führen. Ebenso war die kurze Studiendauer in Anbetracht der spät einsetzenden Wirkung nicht ausreichend, um über die Wirksamkeit abschließend urteilen zu können, weswegen die Erprobung von Sq109 trotz der nicht nachgewiesenen Wirksamkeit weitergeführt wurde.

Die folgende Phase IIB – Studie, PanACEA MAMS TB 01, betreute ich wieder als Sponsor Medical Expert, in Zusammenarbeit mit Prof Hölscher und den akademischen Partnern des PanACEA Konsortiums. (*Boeree, Heinrich et al: High-dose rifampicin, moxifloxacin, and SQ109 for treating tuberculosis: a multi-arm, multi-stage randomised controlled trial* (11)).

Meine Aufgaben lagen in der Entwicklung des Protokolls und der letztendlichen Verantwortlichkeit für das Protokoll, im Design der Datenbank in Zusammenarbeit mit einer Daten-Management Firma, Entwurf des TB Lab Manuals, Überwachung der protokollkonformen Studiendurchführung und Adverse events, wissenschaftlicher Auswertung in Zusammenarbeit mit den Statistikern des Konsortiums, und Publikation.

Diese Studie verwendete ein innovatives Studiendesign mit vier experimentellen Armen; und einem Kontrollarm mit Standardtherapie. Die experimentellen Arme bestanden aus 1. Rifampicin in sehr hoher Dosierung (35mg/kg); mit Isoniazid, Pyrazinamid, Ethambutol in Standarddosis; 2. Rifampicin in mittelhoher Dosierung (20 mg/kg) mit Isoniazid, Pyrazinamid in Standarddosis, und Sq109 300 mg; 3. Rifampicin in mittelhoher Dosierung (20 mg/kg) mit Isoniazid, Pyrazinamid in Standarddosis, und Moxifloxacin 400 mg; und 4. Rifampicin, Isoniazid , Pyrazinamid in Standarddosierungen, mit Sq109 300 mg. Die Zuteilung zu Studienarmen wurde aus den gleichen Gründen wie in der letzten Studie nicht verblindet; allerdings waren die Tb-Labore, die die Daten für den primären Studienendpunkt erhoben, diesbezüglich verblindet.

Die experimentellen Arme wurden für 12 Wochen verabreicht. Der primäre Studienendpunkt war die Zeit bis zum Erreichen einer negativen Kultur innerhalb von zwölf Wochen, welcher mit Cox proportional hazards regression analysiert wurde. Alle Patienten erhielten nach Ende der Intensiv/Experimentellen Therapie Isoniazid und Rifampicin in Standarddosierung, um sechs Monate Tb-Therapie zu komplettieren. Nach Ende der Therapie wurde für sech weitere Monate mittels Telefonanrufen und Ambulanzbesuchen nachverfolgt, um Rezidive diagnostizieren zu können.

Es wurde eine Interims – Analyse eingeplant, um den Patienteneinschluß in Therapiearme, welche die prädefinierte Verbesserung gegenüber der Kontrolle nicht mehr erreichen würden frühzeitig zu stoppen.

1. Zusammenfassung der Habilitationsarbeit

Zwischen 7.5. 2013 und 25.3. 2015 schlossen wir in drei Zentren in Tansania, und vier Zentren in Südafrika 365 Patienten in die Studie ein. Bei der Interimsanalyse wurde der Einschluß in die Arme 2 (Rif 20mg/kg Sq109) und 4 (Rif Standard, Sq109) gestoppt.

Die Zeit bis zur Kulturkonversion im Flüssigmedium war im 35 mg/kg Rif Arm signifikant kürzer als in der Kontrollgruppe (median 48 Tage vs 62 Tage, korrigierte hazard ratio 1·78; 95% CI 1·22–2·58, p=0·003). In den anderen experimentellen Armen zeigte sich kein signifikanter Unterschied. Zudem fanden wir keine signifikanten Unterschiede zwischen den Armen bezüglich der Zeit bis zur Kulturkonversion auf Festmedium (Abbildung 1).

Elf Patienten erlitten ein Therapieversagen oder Rezidiv der Grunderkrankung; einer im 35 mg/kg Rif Arm, drei im Kontrollarm, drei im Arm mit Sq109; vier im 20mg/kg Rif + Sq109 Arm, und kein Patient im 20mg/kg Rif/Moxi arm.
Bei Kontrolle von Verträglichkeit und Toxizität wurden bei 45 (12%) der Patienten unerwünschte Ereignisse der Schweregrade 3 – 5, nach der Schweregrad – Einteilung „Common Toxicity Criteria for Adverse Events 4.0“, mit ähnlichen Verteilung über die Therapiearme.

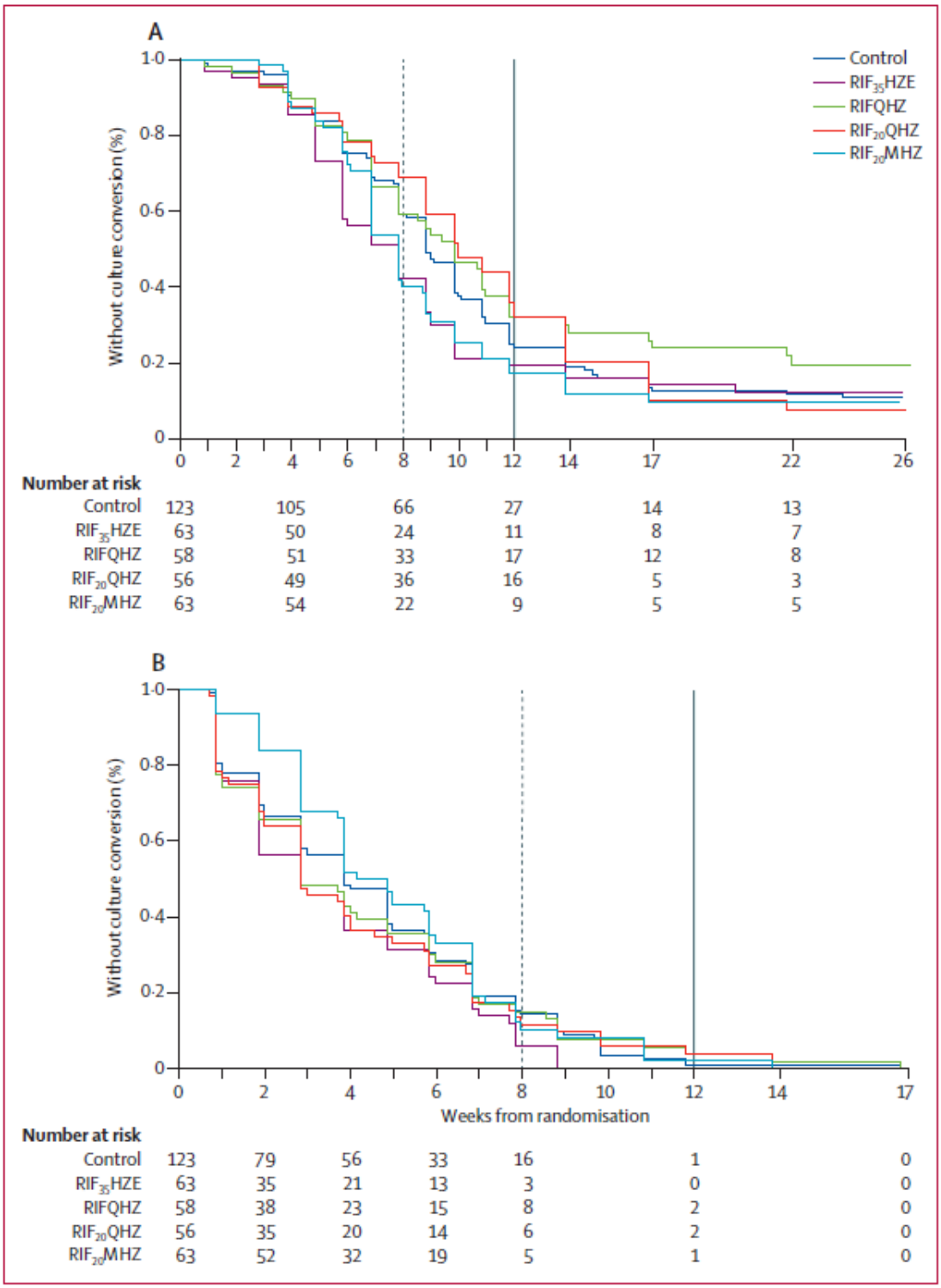

Abbildung 1: Kaplan - Meier Plots zur Zeit bis zur Kulturkonversion A) in Flüssigmedium; und B) auf Festmedium

Wir schlossen aus diesen Ergebnissen, dass die Dosierung von 35 mg/kg Rifampicin täglich, verträglich und sicher war, und wahrscheinlich künftig effektivere und kürzere Tuberkulosebehandlung ermöglichen würde.

Das adaptive Studiendesign wurde erfolgreich angewendet, und kann zukünftig die Entwicklung von neueren Therapien beschleunigen, bei reduzierten Kosten durch die Verwendung eines Kontrollarms für vier experimentelle Arme; und durch frühzeitigen Stopp von nicht erfolgreichen Armen.

Ausblick:

Auf die hier beschriebenen Arbeiten aufbauend, wurde erfolgreich eine zweite Finanzierungsrunde des PanACEA-Konsortiums eingeworben; der folgende kurze Ausblick beschreibt die aktuellen Studien.
Der Wirkstoffkandidat wird BTZ-043 federführend durch die Abteilung für Infektionskrankheiten und Tropenmedizin untersucht; hier leite ich das medical monitoring und begleite die Studie wissenschaftlich.
Gleichzeit bin ich maßgeblich verantwortlich für das Studiendesign, und die Koordination zweier Studien zur Dosisfindung zweier neuer Oxazolidinon - Antibiotika, Sutezolid und Delpazolid, welche die schweren hämatologischen und neurologischen Nebenwirkungen, die vor allem bei längerer Therapie mit Linezolid auftreten, nicht mehr aufweisen sollen. In diesen Studien (PanACEA-SUDOCU-01 und PanACEA–DECODE–01), werden die experimentellen Medikamente in Kombination mit Bedaquilin, Delamanid und Moxifloxazin über 12 bzw. 16 Wochen in verschiedenen Dosierungen gegeben.

Verbessertes Therapiemonitoring

Ein verbessertes Monitoring des Ansprechens von Tuberkulosetherapie ist ein weiteres Thema meiner Arbeit im Rahmen des PanACEA Konsortiums. Derzeit beträgt bei allen Patienten mit nicht-resistenter pulmonaler Tuberkulose die standardisierte Therapiedauer sechs Monate. Aus klinischen Studien ist allerdings bekannt, dass mehr als 80% der Patienten bereits nach vier Monaten dieser Behandlung eine dauerhafte Heilung erzielen würden (12); und dass andererseits auch bei sechs-monatiger Therapie 2 bis 5% einen Rückfall erleiden, d.h. von längerer Therapie profitiert hätten (13).

Aktuell ist es leider nicht möglich, zwischen diesen Patientengruppen zu unterscheiden und eine individuell adaptierte Therapiedauer zu wählen, da kein verläßliches Unterscheidungskriterium existiert. Die Sputum-Mikroskopie nach zwei Monaten Therapie wurde früher als Marker für ein höheres Risiko eines Rückfalls oder Therapieversagens verwendet, um bei anhaltender Positivität die intensive Therapiephase mit vier Medikamenten um einen Monat zu verlängern. Anhand einer großen Studie in Bangladesh wurde jedoch nachgewiesen, dass der positive prädiktive Wert hier bei nur 3% liegt, was entsprechende Übertherapie nach sich zog. Dies läßt sich auch dadurch erklären, dass in der Mikroskopie auch noch durch Therapie abgetötete Bakterien nachgewiesen werden, und das Ergebnis in diesem Fall als falsch positiv zu bezeichnen ist. Die WHO-Strategie, auf Basis der Sputummikroskopie eine Therapieverlängerung zu empfehlen, wurde aufgrund dieser Studienergebnisse beendet.

Die Sputumkultur hat hier einen etwas besseren Vorhersagewert; allerdings beträgt die Sensitivität einer positiven Kultur nach zwei Monaten Therapie zur Detektion eines ungünstigen Langzeitoutcomes nur etwa 52% (14); die Spezifität ist weiterhin niedriger (15). Aufwändigere Kriterien, wie die Zeit bis zum Erreichen einer negativen Kultur, welche in klinischen Studien für neue Therapien verwendet werden, sind zwar geeignet zur Unterscheidung verschiedener Therapien, haben aber leider ebenfalls keine bedeutend höhere Vorhersagekraft bezüglich des individuellen long-term outcome (16). Weiter kompliziert wird die Sputumkultur durch Kontamination, mit zunehmen des Anteils kontaminierter Kulturen mit längerem Therapieverlauf.

In dieser Arbeit beinhaltet sind zwei Publikationen, die auf der Studie “Observation of Early Bactericidal Activity of Standard TB Treatment; OEBA – TB” Studie in Mbeya, Tansania, basieren: *Honeyborne et al: The molecular bacterial load assay replaces solid culture for measuring early bactericidal response to antituberculosis treatment;* (17); und: *Rojas-Ponce et al: A continuously monitored colorimetric method for detection of Mycobacterium tuberculosis complex in sputum* (18)).

Ich war als Leiter der Studie, sowie als Betreuer der PhD-Arbeit, die zur zweiten Publikation führte, beteiligt. Die Studie wurde an Patienten mit neudiagnostizierter pulmonaler Tuberkulose durchgeführt. Vor Beginn der Standardtherapie wurden mindestens zwei Sputen untersucht, danach tägliche Sputen während der ersten zwei Therapiewochen. Die Bakterienlast wurde durch Kultur mehrerer Verdünnungsstufen auf 7H11 – Festmedium und Auszählen der gebildeten Bakterienkolonien bestimmt, um die Zahl der koloniebildenden Einheiten (CFU) zu bestimmen. Ein Teil

des Sputums wurde in BD MGIT Flüssigmedium inokuliert, hier wurde die Zeit bis zur Detektion von Wachstum (*time to positivity*, TTP) als Marker für die Bakterienlast verwendet.

Die erste Arbeit befasste sich mit einem direkten Vergleich des molecular bacterial load assay (MBL) mit Kultur. MBL ist ein molekularer Assay, der eine quantitative Abschätzung der Bakterienlast im Sputum auf Basis von Analyse der bakteriellen 16S RNA und eines internen Standards liefert. Der Assay wurde von Kollaborationspartnern am University College of London (UCL) entwickelt.

Die hier publizierte Validierung des MBL wurde an Patienten der OEBA-TB Studie durchgeführt. Aus Sputen, die innerhalb der ersten 14 Tage abgegeben wurden, wurde sowohl eine quantitative Kultur auf Flüssig- und Festmedium durchgeführt, als auch der MBL Assay.

Es zeigte sich eine gute Korrelation für individuelle Sputen zwischen MBL und sowohl Fest- (Spearman rho = 0,84; 95% CI 0,78 – 0,88) als auch Flüssigmedium (Spearman rho = -078; 95% CI -0,84 - -0,71).

Ebenso zeigten sich gute Korrelationen für die Änderung der Bakterienlast unter Therapie, sowohl für individuelle Patienten, als auch für die gesamte Kohorte. Zu Beginn der Therapie lag die Bakterienlast im MBL bei 0,43 $\log_{10}$CFU (95% CI 0,12 – 0,73), also höher als in der Kultur auf Festmedium, was wahrscheinlich auf eine gewisse Hemmung des Bakterienwachstums im durch die im Festmedium enthaltenen Antibiotika bedingt ist - dies konnte auch in einem in vitro Ansatz nachvollzogen werden. Die Abnahme der Bakterienlast zwischen Tag 3 und 14 der Therapie wurde im Festmedium mit 0,082 $\log_{10}$CFU/d (95% CI 0,045 – 0,119) und im MBL mit 0,124 $\log_{10}$/d (95% CI 0,170 - 0,079) quantifiziert. Die biphasische Kinetik mit stärkerer Abnahme während der ersten drei Therapietage, die ein typisches Merkmal der Standard-TB-Therapie ist, wurde in allen drei Medien gut wiedergegeben.

Die intra-individuelle Variation der quantitativen Bakterienlast war im MBL mit 9,6% Varianz zwischen beiden baseline samples am geringsten, gefolgt von 17,9% für Flüssig - und 21,6% für Festmedium. Kontamination und andere Gründe führten zu einem fehlenden Ergebnis in 1,2 % aller Proben im MBL, und 11 % aller Proben auf Festmedium.

In der Zusammenschau zeigte sich in dieser Studie, das der MBL ein vielversprechender molekularer Assay ist, der was Präzision, Zeit bis zur Verfügbarkeit des Ergebnisses, und Ausfallshäufigkeit angeht der Kultur überlegen ist. Der Assay wird derzeit an archivierten Proben der größeren PanACEA – MAMS – TB-01 Studie erprobt, und wird danach vermutlich erstmalig während einer Studie als Studienendpunkt zur Messung des Therapieerfolgs mitgeführt werden.

STC-NRA

Eine weitere Arbeit befasst sich mit der Evaluation einer vereinfachten colorimetrischen Kulturmethode zum (quantitativen) Nachweis von *M.tuberculosis* Komplex (MTB; (18). Die aktuell verwendete kommerzielle „Mycobacterium Growth Indicator Tube“ (MGIT) der Firma Becton Dickinson wird vor allem in wirtschaftlich benachteiligten Gesundheitssystemen wegen hoher Kosten und Anforderungen an Elektriziät, Personal, Reagenzienversorgung und Nachtestung zur Speziesbestimmung, die in einem BSL 3- Labor stattfinden muss, kaum verwendet. Traditionell greifen daher vor allem südamerikanische Länder auf Flüssigkulturmethoden wie MODS (microscopic observation drug susceptibility assay) oder den Nitratreduktaseassay (NRA) zurück.

Klassische kolorimetrische Kulturmethoden leisten eine Unterscheidung zwischen Spezies des humanpathogenen MTB, und den meisten der nicht- oder fakultativ pathogenen nicht-tuberkulösen Mykobakterien (NTM). Die meisten NTMs testen zwar auch positiv im Nitratreduktaseassay, durch P-Nitrobenzoesäure (PNB) ist aber eine selektive Wachstumshemmung von MTB, nicht aber NTM (19), möglich, welche zur Unterscheidung herangezogen werden kann.

Der Nachteil klassischer kolorimetrischer Methoden liegt in der Notwendigkeit der mehrfachen Untersuchung der Kultur zur Feststellung von Wachstum, bevor der Nitratreduktasetest durchgeführt werden kann. Dies ist arbeitsintensiv und beinhaltet die Gefahr der Bildung infektiöser Aerosole und von Kontamination bei wiederholter Öffnung der Kultur.

1. Zusammenfassung der Habilitationsarbeit

In der vorliegenden Studie wurde der Redox-Indikator 2,3-diphenyl-5-thienyl-(2)-tetrazoliumchlorid (STC) verwendet, welcher im Medium verbleiben kann und durch Farbumschlag ein Wachstum anzeigt. Drei Kulturansätze pro Probe sind notwendig, welche in der vorliegenden Arbeit mit einem Volumen von jeweils 1ml 7H9 Flüssigmedium sehr klein dimensioniert wurden.

Ein Tube (C) wird mit STC zur Kontrolle von Wachstum versetzt, ein Tube (N) ohne Zusätze zur Durchführung des Nitratreduktasetests bei Nachweis von Wachstum in Tube C, sowie ein Tube (NP) mit PNB zur Durchführung des Nitratreduktasetests; in diesem Tube wird Wachstum von MTB, nicht aber NTM, selektiv von PNB gehemmt. Der Ansatz wird wöchentlich kontrolliert, bei Farbumschlag in Tube C wird der NRA durchgeführt. Dieser experimentelle Ansatz wird mit „STC-NRA“ bezeichnet.

In der Arbeit wurden 93 Sputen von 18 OEBA-TB Patienten vor und unter Therapie sowohl im BD MGIT als Standardmethode, als auch mit der experimentellen STC-NRA Methode untersucht. 12 von 93 (13%) Proben im STC-NRA, sowie 29 von 93 (31%) Proben im MGIT zeigten Kontamination, und damit keine verwertbaren Ergebnisse.

47 Proben im STC-NRA, und 59 im MGIT, zeigten Wachstum von MTB. 8 Proben im STC-NRA zeigten Wachstum von Mykobakterien, die mittels PNB nicht zu klassifizieren waren. Keine Probe war positiv für NTM.

Für 60 Kulturen war sowohl im MGIT, als auch im STC-NRA eine Zeit bis zum Nachweis von Wachstum als quantitativer Marker der Bakterienlast verfügbar. Im Median waren dies 14 Tage im STC-NRA, und 7 Tage im MGIT. Beide Methoden zeigten mit zunehmender Therapiedauer eine ansteigende Zeit bis zum Nachweis von Wachstum, was mit einer abnehmenden Bakterienzahl im Sputum korreliert (20).

Zusamenfassend zeigt diese Studie, dass der STC-NRA eine relativ einfache, kostengünstige Alternative zur kommerziellen Flüssigkultur darstellt. Unter der Annahme, dass die 8 Proben mit unklassifizierbaren Mykobakterien tatsächlich Wachstum von MTB anzeigten, die aufgrund der Therape ihre NRA-Positivität verloren, unterscheiden sich die Nachweisraten von MTB in beiden Systemen nicht; STC-NRA war jedoch wesentlich weniger anfällig für Kontamination. Die längere Zeit bis zum Nachweis von Wachstum in STC-NRA ist wahrscheinlich durch das kleinere Inokulum, das nur 1/5 des Volumens im MGIT betrug, bedingt.

Ausblick:

In den oben beschriebenen Studien zur Evaluation von BTZ-043, aber auch SUDOCU und DECODE, wird der MBL Assay aktuell unter meiner Leitung weiter evaluiert bezüglich der Möglichkeit, die Kultur als Biomarker in TB-Therapiestudien zu ersetzen. Weiterhin konnten wir durch Aufarbeitung von Proben aus der PanACEA-MAMS-01 Studie zeigen, dass mittels MBL Assay ungünstige Verläufe früher erkannt werden können, als mittels der MGIT Flüssigkultur (Ntinginya, eingereicht zur Publikation).

Im Jahr 2021 wurde unter Leitung von Prof. Hölscher eine umfassende Finanzierung der Innovative Medicines Initiative (IMI) für das Unite4TB- Konsortium eingworben. Hier wird eine große Zahl weiterer Studien zu neuen TB-Medikamenten durchgeführt werden, ich bin in diesem Konsortium als einer von zwei Leitern der Evaluation neuer Biomarker aktiv.

Einige der als Biomarker evaluierten Assays sind ebenfalls als diagnostische Tests für Tuberkulose nutzbar. Hier gelang es mir, Finanzierung in Höhe von 3 Mio € für eine große klinische Studie („Rapid and accurate diagnosis of paediatric TB, RaPaed-TB“, rapaed.org) zur Validierung als Diagnostika bei Kindern einzuwerben – die Diagnosefindung vor allem bei kleinen Kindern ist schwieriger als die bei Erwachsenen, da Kinder nicht auf Anforderung gute Sputumproben abgeben können, und die Erkrankung bei Kindern durch geringere Bakterienlast in Sekreten schwerer nachzuweisen ist.

Die RaPaed-Studie hat aktuell den Patienteneinschluß beendet, und ist mit 974 Kindern eine der größten derartigen Kohorten.

Ein weiteres diagnostisches Problem wird in dem von mir geleiteten ERASE-TB Konsortium untersucht. Die von mir eingeworbene Finanzierung in Höhe von 3,3 Mio € erlaubt hier die Validierung von neuen

1. Zusammenfassung der Habilitationsarbeit

Tests zur Frühdiagnose von Tuberkulose bei Haushaltskontakten von hochinfektiösen Indexfällen. Diese Studie begann im Frühjahr 2021, und soll 2,100 Haushaltskontakte einschließen.

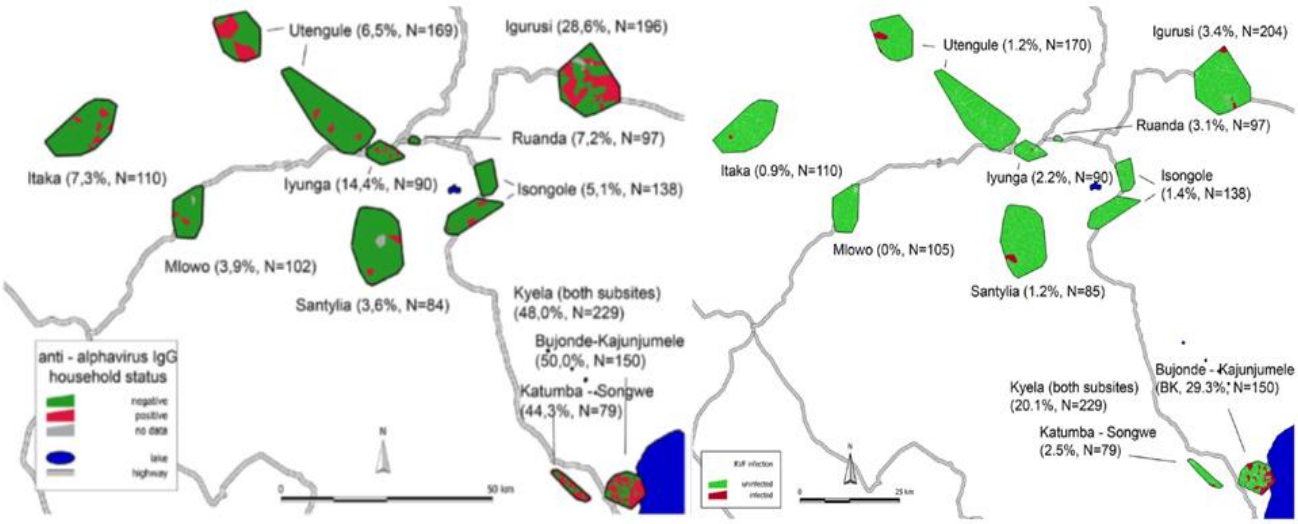

Abbildung 2: geografische Verteilung der Haushalte mit mindestens einem seropositiven Teilnehmer für Alphavirus (links) und Rifttalfieber (rechts).

Vernachlässigte, durch Arthropoden übertragene Erkrankungen in Südwest-Tansania

Die hier vorgestellten Arbeiten stammen aus einer Reihe von seroepidemiologischen Untersuchungen von Teilnehmern der EMINI (Evaluation and Monitoring of the Impact of New Interventions) – Bevölkerungskohortenstudie. Diese Studie beobachtete insgesamt 17.872 Teilnehmer aus neun Orten in der Region Mbeya, im Südwesten Tansanias, über 5 Jahre. Die Orte wurden ausgewählt um das Spektrum sozio-ökonomischer und ökologischer Bedingungen der Region zu repräsentieren. Es wurden Daten sowohl zur eigenen Gesundheit, als auch zu sozio-ökonomischen Aspekten erhoben, und die geographische Position des Haushalts mit GPS vermessen.

Durch randomiserte stratifizierte Selektion wählten wir aus den im Jahr 2007 abgenommenen Proben 1.228 Proben aus. Im Rahmen von zwei medizinischen Dissertationen wurden an diesen Proben serologische Querschnittsuntersuchungen durchgeführt. Korrelationen von Seropositivität mit sozio-ökonomischen und ökologischen Variablen wurden untersucht, um einen Aufschluß über Risikofaktoren und mögliche Infektionswege zu erhalten. Es handelte sich mit Rifttalfieber (RTF) und *Alphavirus*-Erkrankungen um zwei durch Moskitos übertragene Erkrankungen, wobei das Chikungunya-Virus das am weitesten verbreitete *Alphavirus* ist. Weiterhin wurden Rickettsien sowohl der Zeckenbiß-Fleckfieber – als auch der epidemischen Fleckfiebergruppe untersucht.

Erreger, die durch Moskitos übertragen werden: Rifttalfieber (RFT) und *Alphavirus*

Aus dieser In der Serologie zeigten sich im Gesamtkollektiv 5,2% positiv für IgG gegen RTF, und 18% positiv für IgG gegen Alphavirus. (*Heinrich et al: High seroprevalence of rift valley Fever and evidence for endemic circulation in mbeya region, Tanzania, in a cross-sectional study* (21); *Weller et al: Seroprevalence of Alphavirus Antibodies in a Cross-Sectional Study in Southwestern Tanzania Suggests Endemic Circulation of Chikungunya* (22)).

Die Verteilung der Seroprävalenzen in den neun Studienorten war besonders für RTF inhomogen. Der Ort der höchsten Prävalenz war Bujonde – Kajunjumele (BK) am Ufer des Malawisees, mit 29,3% Seroprävalenz für RTF und 50,0 % für *Alphavirus*. Ein Vorkommen von RTF- Seropositivität ausserhalb von BK wurde kaum nachgewiesen, mit 3,4% als zweithöchster Seroprävalenz in Igurusi. *Alphavirus*-Seropositivität zeigte eine homogenere Verteilung im Studiengebiet, die eher linear mit der Höhenlage des Wohnortes korrelierte.

Bei beiden Serologien zeigte sich eine fast lineare Abhängigkeit der Seroprävalenz vom Alter der Studienteilnehmer, was auf ein eher kontinuierliches Infektionsrisiko, mit kumulativ steigendem Lebenszeitrisiko bei endemischen Erkrankung schließen lässt, im Gegensatz zu epidemischem Auftreten.

Risikofaktoren für RTF-Seroprävalenz wurden durch Poisson-Regression ermittelt. Niederiger sozio-ökonomischer Status (Prävalenzratio PR 0,60 pro Punkt Anstieg des sozio-ökonomischen Status-Scores, 95% CI 0,40 – 0,90), der Besitz von Rindern (PR 1,81; 95% CI 1,15-2,85) und höhere Vegetationsdichte (PR 2,99 pro Zunahme des Enhanced Vegetation Index um einen Punkt; 95% CI 1,34 – 6,65) waren klar mit Seropositivität assoziiert. Für eine Reihe von nicht-unabhängigen ökologischen Faktoren zeigte sich eine Assoziation mit Seropositivität, so waren Abstand zum Malawisee (PR 0,79 pro km, 95% CI 0,69 – 0,90), höhere Durchschnittstemperatur (PR 0,87 pro °C, 95% CI 0,81 – 0,94) und Höhenlage (PR 0,87 pro m; 95% CI 0,80 bis 0,94) negativ; und höhere Mindesttemperatur positiv (PR 2,51 pro °C, 95% CI 0,94-6,7) mit Seroprävalenz assoziiert.

Wahrscheinlich ist der kausale Zusammenhang zwischen ökologischen Faktoren und Seropositivität hauptsächlich auf saisonale Überschwemmungen zurückzuführen, welche in der Nähe des Malawisees auftreten, den Überträgermücken Brutplätze bieten und tatsächlich bei Anstieg der Höhenlage um wenige Meter nicht mehr relevant sind.

Bei der Ermittlung von Risikofaktoren für die *Alphavirus*-Seroprävalenz zeigte sich ein Zusammenhang mit der Höhenlage des Wohnorts, mit medianen Höhenlagen zwischen 478 m für Kyela und 2018 m für Santylia; und deutlich niedrigeren Seroprävalenzen oberhalb von 1290 m. Ebenso zeigte sich die hier erstmalig einbezogene Hangneigung des Geländes als relevant, mit einer PR von 0,86 pro Grad zusätzlicher durchschnittlicher Hangneigung (95% CI 0,77 – 0,95), die wahrscheinlich mit weniger stehenden Kleingewässern (Pfützen), die als Moskitobrutplatz dienen, assoziiert ist.

Im Vergleich beider Serologien zeigte sich eine wesentlich geringere Ausbreitung von RTF im Studiengebiet, praktisch ausschließlich am Ufer des Malawisees oder der zuführenden Flüsse; während *Alphavirus* weiter verbeitet ist, und wahrscheinlich nur durch die Höhenlage und Verfügbarkeit von stehendem Oberflächenwasser als Vektorbrutplatz limitiert wird.

Rickettsiosen

Unter Rickettsien der alten Welt können serologisch zwei Gruppen dieser obligat intrazellulären Erreger unterschieden werden: die epidemischen Fleckfieber (engl. Typhus) – Gruppe , mit den Spezies *Rickettsia typhi* und *R. prowazekii*; und Rickettsien der Zeckenbiß- Fleckfieber (engl. Spotted Fever) – Gruppe, mit den Spezies *R. conorii, R. africae*, und anderen (23).

In der ersten Arbeit analysierten wir die Seroprävalenz der epidemischen Fleckfiebergruppe und assoziierte Risikofaktoren in der Studienpopulation (*Dill et al: High seroprevalence for typhus group rickettsiae, southwestern Tanzania* (24); *Heinrich et al: High seroprevalence for spotted Fever group rickettsiae, is associated with higher temperatures and rural environment in mbeya region, southwestern Tanzania* (25).

Es zeigte sich eine Prävalenz von 9,3%, mit einem lokalen Maximum in den urban geprägten Studienorten Ruanda (17,5%) und Iyunga (17,2%), beide Mbeya- Stadt, und mit Minima in den ländlichen Studienorten Itaka (2,7%) und Kyela (5,2%).

Im multi-variablen Modell fanden wir signifikante Korrelationen zwischen Seroprävalenz und mittlerem bis höherem Lebensalter, Abstand zur nähesten Fernstraße (PR 0,97 pro km, 95% CI 0,46 – 0,79), und niedriger Vegetationsdichte (PR 0,60 pro Zunahme des Enhanced Vegetation Index um 0,1 Punkte; 95% CI 0,46-0,79). Interessanterweise waren die Variablen „Bevölkerungsdichte“ und „Vegetationsdichte“ in der uni-variablen Analyse signifikant mit Seropositivität assoziiert, und miteinander korreliert. In der multi-variablen Analyse mit beiden Variablen war die Assoziation mit „Bevölkerungsdichte“ nicht signifikant, während die Vegetationsdichte als offensichtlich besser korrelierte Variable signifikant assoziiert blieb.

Serologisch ist eine durchgemachte Infektion mit *R. typhi* nicht von *R.prowazekii* zu unterscheiden. Da epidemische Fleckfiebererkrankungen mit hoher Sterblichkeit, wie durch *R. prowazekii* ausgelöst, unseres Wissens im Studiengebiet nicht vorkommen, liegt die Annahme nahe, dass es sich um durch *R. typhi* oder eine bisher unbekannte Spezies ausgelöste Erkrankungen mit eher mildem Verlauf handelt.

In der Literatur bestätigt sich ein vermehrtes Auftreten von endemischem Fleckfieber durch R. typhi, in Städten, insbesondere Hafenstädten (26, 27) wie Dar es Salaam. Es ist hierbei anzunehmen, dass, wie in anderen Settings, Ratten das Erregerreservoir darrstellen, und der Rattenfloh *Xenopsylla cheopsis* als Vektor für die Übertragung auf den Menschen verantwortlich ist. Die höhere Seroprävalenz in städtischen Gebieten unserers Untersuchungsgebietes unterstützt diese Vermutung.

Die Untersuchung der Zeckenbiß-Fleckfiebergruppe (spotted fever group) zeigte eine deutliche höhere Seroprävalenz von 67,9%.

Die höchste Seroprävalenz wurde mit 91,4% in Mlowo, einem Ort mittlerer Höhenlage, eher dichterer Bevölkerung und hoher Anzahl an Rindern gefunden. Das finale multi-variable Modell aller Kofaktoren zeigte eine positive Assoziation der Seroprävalenz mit steigendem Lebensalter (PR 1,08 bei Zunahme um 10 Jahre, 95% CI 1,06 – 1,10); männlichem Geschlecht ((PR 1,08; 95% 1,00 – 1,16); höherern Durchschnittstemperatur (PR 1,31 pro °C, 95% CI 1,05 – 1,64) und niedriger Bevölkerungsdichte (PR 0,93 pro Zunahme um 1,000 Personen/km²; 95% CI 0,90 - 0,96). Die höchsten Seroprävalenzen wurden unterhalb von 1,578 Höhenmeter gemessen. Eine positive Interaktion mit der Dichte an Rindern pro Quadratkilometer war in Höhenlagen über 1,291 m gegeben, d.h. dass in den höheren Lagen die Seropositivität signifikant mit der Rinderdichte korreliert.

Zusammenfassend wurde in unserem Kollektiv eine ähnlich hohe Seroprävalenz wie in anderen Settings in sub-Sahara Afrika gefunden (28, 29). Nachdem serologisch keine Differenzierung der Erreger möglich war, erlauben die Assoziationen in der Auswertung die Vermutung, dass es sich hier vornehmlich um afrikanisches Zeckenbißfieber handelt, ausgelöst durch *R. africae* - nicht um Mittelmeer-Fleckfieber (*R. conorii*). Höhere Temperaturen und niedrigere Höhenlage begünstigen generell die Übrtragung durch wechselwarme Vektoren, allerdings deutet die Assoziation mit ländlicheren, bevölkerungsarmen Gebieten, und der Dichte an Rindern auf eine Rinderzecke wie die in Tansania heimische *Amblyomma variegatum* hin (30) – das Infektionsrisiko mit *R. conorii*, übrtragen durch die Hundezecke *Rhipizephalus sanguineus*, ist üblicherweise in städtischen Gebieten höher.

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High-dose rifampicin, moxifloxacin, and SQ109 for treating tuberculosis: a multi-arm, multi-stage randomised controlled trial.

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High-dose rifampicin, moxifloxacin, and SQ109 for treating tuberculosis: a multi-arm, multi-stage randomised controlled trial

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Summary

Background Tuberculosis is the world's leading infectious disease killer. We aimed to identify shorter, safer drug regimens for the treatment of tuberculosis.

Methods We did a randomised controlled, open-label trial with a multi-arm, multi-stage design. The trial was done in seven sites in South Africa and Tanzania, including hospitals, health centres, and clinical trial centres. Patients with newly diagnosed, rifampicin-sensitive, previously untreated pulmonary tuberculosis were randomly assigned in a 1:1:1:1:2 ratio to receive (all orally) either 35 mg/kg rifampicin per day with 15–20 mg/kg ethambutol, 20 mg/kg rifampicin per day with 400 mg moxifloxacin, 20 mg/kg rifampicin per day with 300 mg SQ109, 10 mg/kg rifampicin per day with 300 mg SQ109, or a daily standard control regimen (10 mg/kg rifampicin, 5 mg/kg isoniazid, 25 mg/kg pyrazinamide, and 15–20 mg/kg ethambutol). Experimental treatments were given with oral 5 mg/kg isoniazid and 25 mg/kg pyrazinamide per day for 12 weeks, followed by 14 weeks of 5 mg/kg isoniazid and 10 mg/kg rifampicin per day. Because of the orange discoloration of body fluids with higher doses of rifampicin it was not possible to mask patients and clinicians to treatment allocation. The primary endpoint was time to culture conversion in liquid media within 12 weeks. Patients without evidence of rifampicin resistance on phenotypic test who took at least one dose of study treatment and had one positive culture on liquid or solid media before or within the first 2 weeks of treatment were included in the primary analysis (modified intention to treat). Time-to-event data were analysed using a Cox proportional-hazards regression model and adjusted for minimisation variables. The proportional hazard assumption was tested using Schoelfeld residuals, with threshold p<0·05 for non-proportionality. The trial is registered with ClinicalTrials.gov (NCT01785186).

Findings Between May 7, 2013, and March 25, 2014, we enrolled and randomly assigned 365 patients to different treatment arms (63 to rifampicin 35 mg/kg, isoniazid, pyrazinamide, and ethambutol; 59 to rifampicin 10 mg/kg, isoniazid, pyrazinamide, SQ109; 57 to rifampicin 20 mg/kg, isoniazid, pyrazinamide, and SQ109; 63 to rifampicin 10 mg/kg, isoniazid, pyrazinamide, and moxifloxacin; and 123 to the control arm). Recruitment was stopped early in the arms containing SQ109 since prespecified efficacy thresholds were not met at the planned interim analysis. Time to stable culture conversion in liquid media was faster in the 35 mg/kg rifampicin group than in the control group (median 48 days *vs* 62 days, adjusted hazard ratio 1·78; 95% CI 1·22–2·58, p=0·003), but not in other experimental arms. There was no difference in any of the groups in time to culture conversion on solid media. 11 patients had treatment failure or recurrent disease during post-treatment follow-up: one in the 35 mg/kg rifampicin arm and none in the moxifloxacin arm. 45 (12%) of 365 patients reported grade 3–5 adverse events, with similar proportions in each arm.

Interpretation A dose of 35 mg/kg rifampicin was safe, reduced the time to culture conversion in liquid media, and could be a promising component of future, shorter regimens. Our adaptive trial design was successfully implemented in a multi-centre, high tuberculosis burden setting, and could speed regimen development at reduced cost.

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Introduction

Tuberculosis is now the leading infectious disease killer worldwide. Treatment regimens last at least 6 months, so shorter, safer, and more effective regimens for drug-sensitive tuberculosis are needed as part of the global strategy to eliminate the disease.

Rifampicin is a key drug that, combined with pyrazinamide, reduced tuberculosis treatment from 18 to 6 months.[1,2] The standard dose of rifampicin (10 mg/kg) was chosen in the 1960s, primarily because of cost.[2] However, results of several studies in mice[3–6] showed that higher doses can accelerate cure, and

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See Online for appendix

Research in context

Evidence before this study

We did literature searches in PubMed, applying the Medical Subject Heading (MeSH) terms "tuberculosis"; and "rifampicin AND dose", "moxifloxacin", or "SQ109". Publications on pulmonary tuberculosis listed not later than April 30, 2016, in English, German, French, and Italian language were considered.

Pharmacokinetic studies indicate that standard dose (1 0mg/kg) of rifampicin often did not achieve effective plasma concentrations in patients. Enhanced efficacy of higher doses of rifampicin was reported in a number of animal studies. A systematic review on elevated doses of rifampicin published before 2008 identified 14 studies testing up to 1200 mg of rifampicin. Despite difficulties comparing efficacy outcomes across trials, there was an indication that higher doses were beneficial. Among 339 articles published after 2008, a single 14-day dose-ranging study of up to 35 mg/kg reported a supra-proportional increase in pharmacokinetic parameters and good tolerability, with a suggestion of enhanced early bactericidal activity. 43 publications on SQ109 were identified. A mouse study reported improved efficacy when SQ109 replaced ethambutol, but with a delayed onset of several weeks in reduction of lung and spleen colony-forming units. One 14-day phase 1 and one phase 2a study reported good tolerability, but absence of early bactericidal activity during the 14-day phase 2a study duration. A meta-analysis of moxifloxacin trials concluded that this drug added to the bactericidal activity of the regimen when it replaced ethambutol or isoniazid, but this was not enough to shorten treatment duration from 6 to 4 months.

Added value of this study

This study showed that 35 mg/kg rifampicin given over 12 weeks was safe and shortened the time to stable culture conversion from 62 to 48 days, showing the potential for an enhanced regimen. The other experimental arms, including various combinations of 10 mg/kg or 20 mg/kg of rifampicin, moxifloxacin, and SQ109, did not achieve significant improvements over the control arm. Taking all the data into consideration, this study supports that rifampicin given at 35 mg/kg is likely to improve treatment outcome. To our knowledge, this is the first time that a multi-arm adaptive trial design was successfully implemented in a multi-centre study in a high tuberculosis burden setting. This approach might accelerate tuberculosis regimen development at a reduced cost.

Implications of all the available evidence

Our study substantiated that an increase in rifampicin dose could improve the clearance of bacteria in patients with pulmonary tuberculosis without an increase in associated adverse events. The scale of the improvement shown in this study of 35 mg/kg rifampicin administered orally could translate to improved clinical outcomes. Smaller increases in rifampicin doses did not have this effect and suggest that future pivotal phase 3 studies should be done with at least 35 mg/kg.
The combination of moxifloxacin and 20 mg/kg rifampicin had a modest effect on bacterial clearance. This combination could be improved by increasing the dose of moxifloxacin to overcome induction of its metabolism by rifampicin.

higher doses seemed to increase sputum culture conversion in clinical trials.[7] In a dose ranging trial,[8] 35 mg/kg showed increased efficacy and good tolerability when administered daily for 14 days. In another phase 2 study[9] of 600 mg (10 mg/kg), 900 mg (15 mg/kg), and 1200 mg (20 mg/kg) rifampicin with standard concomitant treatment, patients showed good tolerability but no difference in efficacy in the three groups.

To address the challenge of choosing among the many potential drug combinations that should be assessed in phase 3 clinical trials, we adapted a multi-arm, multi-stage trial design[10] that has been used successfully in oncology,[11] and in which multiple regimens are compared with a common standard regimen. Recruitment to insufficiently efficacious regimens is discontinued if prespecified thresholds are not achieved to save time and resources, and to reduce the risk of exposing patients to an ineffective treatment. The objective of this approach is to generate data to select a regimen that might be eligible to progress to a pivotal phase 3 trial. We selected regimens based on literature and on previous studies within our consortium PanACEA (Pan African Consortium for the Evaluation of Antituberculosis Antibiotics). Regimens assessed in our trial also included 300 mg SQ109 (Sequella, Rockville, MD), a well-tolerated drug candidate based on the ethylene diamine pharmacopore.[12,13] Moxifloxacin was chosen to be assessed in combination with 20 mg/kg rifampicin. Moxifloxacin is a licensed antibiotic that leads to faster culture conversion when substituted for either isoniazid or ethambutol.[14–16]

We present the results of the PanACEA MAMS-TB trial, in which we aim to investigate four new potential regimens and establish a new pathway for tuberculosis drug regimen development.

Methods

Study design

We did a randomised, controlled, open-label, multi-arm, multi-stage study (MAMS) in three clinical trial sites in Tanzania and four trial sites in South Africa, including hospitals, health centres, and clinical trial units. The protocol was approved by independent ethics committees of the sponsor and the trial sites, and regulatory authorities of Tanzania and South Africa, and done according to Good Clinical Practice guidelines.[17] The protocol is available in the appendix and panacea-tb.net.

Patients

Eligible patients were aged 18 years or older, weighed 35–90 kg, had newly diagnosed, previously untreated pulmonary tuberculosis confirmed to be rifampicin sensitive by Xpert MTB/RIF, and positive smear microscopy of at least 1+ on the IUATLD/WHO scale. Patients with HIV were eligible if their CD4 count was greater than 200 cells per μL, and where local ethics committees agreed that antiretroviral treatment could be safely withheld until study week 12. Female patients were excluded if they were pregnant or breastfeeding. Patients were excluded if they received or required therapy expected to prolong the QT interval in electrocardiogram (ECG), or alter cytochrome P450 enzyme activity with potential effects on SQ109 metabolism (appendix p 7). Patients were recruited by invitation if diagnosed with tuberculosis in the public health system and included if they gave written informed consent

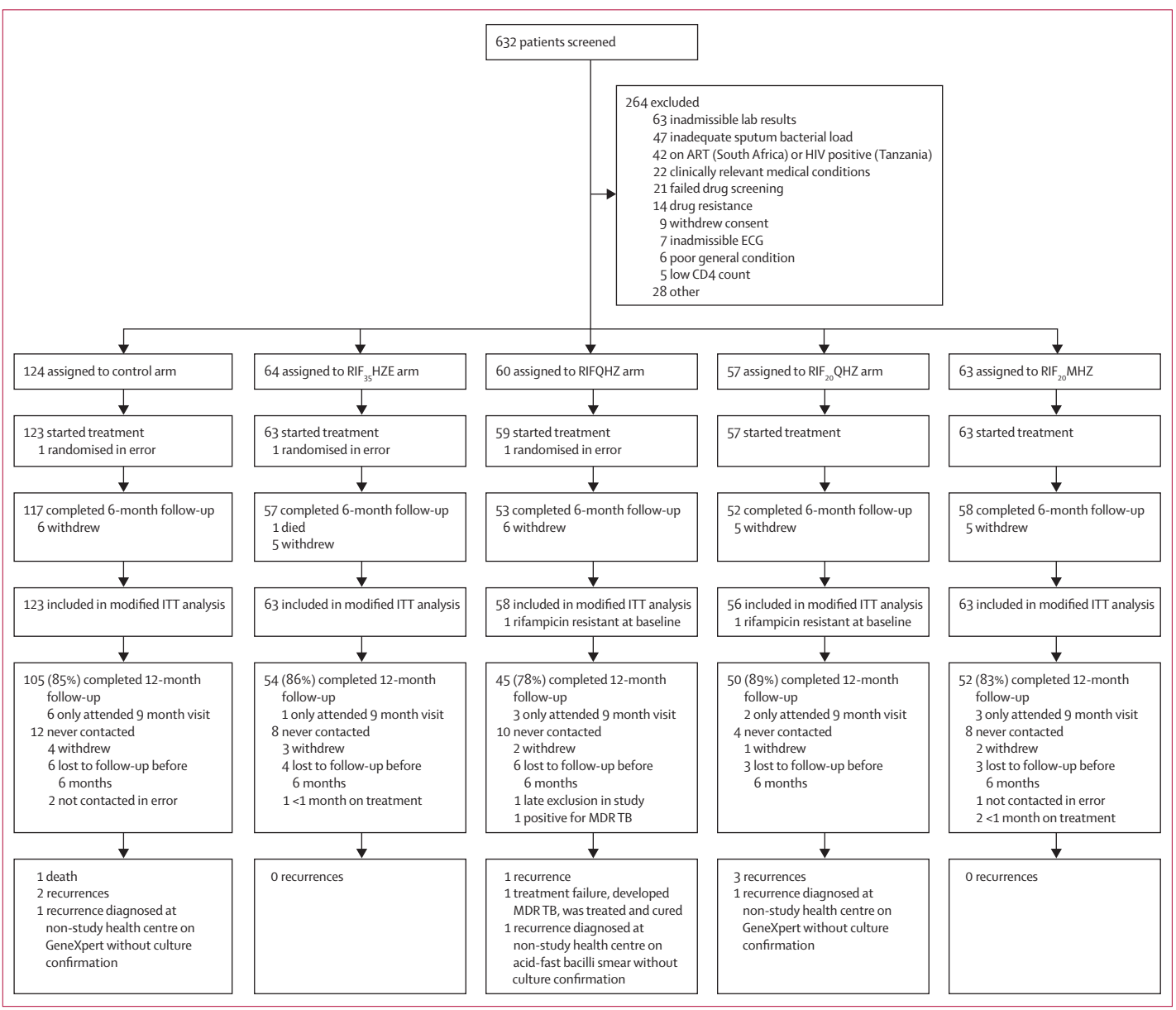

Figure 1: **Trial profile**

Recruitment to RIFQHZ arm and $RIF_{20}QHZ$ arm was stopped early following the first interim analysed. Three patients who were randomised in error were not started on treatment and not retained in follow-up. $RIF_{35}HZE$=rifampicin 35 mg/kg, isoniazid, pyrazinamide, ethambutol. RIFQHZ=rifampicin 10 mg/kg, isoniazid, pyrazinamide, SQ109 300 mg. $RIF_{20}QHZ$=rifampicin 20 mg/kg, isoniazid, pyrazinamide, SQ109 300 mg. $RIF_{20}MHZ$=rifampicin 20 mg/kg, isoniazid, pyrazinamide, moxifloxacin 400 mg. ITT=intention-to-treat. MDR TB=multi-drug resistant tuberculosis. Doses of concomitant drugs are detailed in Procedures.

Randomisation and masking

Patients were randomly assigned centrally using a web-based computerised algorithm, developed and maintained by the MRC Clinical Trials Unit. We used a random element of 80% for minimisation, stratified on study site, baseline bacterial load reported by Xpert MTB/RIF (high *vs* low; high is <16 cycle threshold), and HIV status. Eligible patients were assigned in a 1:1:1:1:2 ratio to one of four daily experimental regimens or a daily control regimen. Because of the orange discoloration of body fluids with higher doses of rifampicin it was not possible to mask patients and clinicians to treatment allocation. However, all laboratory assessments were done blind to treatment allocation and only the independent data monitoring committee (IDMC) and the trial statisticians saw data aggregated by treatment arm during the trial.

	Control	$RIF_{35}HZE$	RIFQHZ	$RIF_{20}QHZ$	$RIF_{20}MHZ$	Total
Number randomised*	123	63	59	57	63	365
Age (years)	34 (26–41)	33 (23–40)	32 (25–40)	34 (27–41)	31 (24–38)	33 (26–40)
Male	94 (76%)	42 (67%)	38 (64%)	45 (79%)	39 (62%)	258 (71%)
Weight (Kg)	54 (49–59)	52 (47–58)	53 (47–57)	53 (49–56)	52 (48–61)	53 (49–58)
HIV positive	9 (7%)	4 (6%)	5 (8%)	3 (5%)	3 (5%)	24 (7%)
Ethnicity						
Black	101 (82%)	51 (81%)	50 (85%)	50 (88%)	48 (76%)	300 (82%)
White	0 (0%)	1 (2%)	0 (0%)	1 (2%)	0 (0%)	2 (1%)
Mixed	19 (15%)	11 (17%)	9 (15%)	6 (11%)	15 (24%)	60 (16%)
Other	3 (2%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	3 (1%)
Xpert MTB/RIF cycle threshold	16 (14–19)	17 (14–20)	17 (14–19)	16 (14–18)	16 (14–19)	16 (14–19)
Phenotypic resistance to rifampicin						
Resistant	0 (0%)	0 (0%)	1 (2%)	1 (2%)	0 (0%)	2 (1%)
Sensitive	112 (91%)	59 (94%)	54 (92%)	53 (93%)	56 (89%)	334 (92%)
Missing	11 (9%)	4 (6%)	4 (7%)	3 (5%)	7 (11%)	29 (8%)
Phenotypic resistance to isoniazid						
Resistant	3 (2%)	0 (0%)	3 (5%)	1 (2%)	1 (2%)	8 (2%)
Sensitive	109 (89%)	59 (94%)	52 (88%)	53 (93%)	55 (87%)	328 (90%)
Missing	11 (9%)	4 (6%)	4 (7%)	3 (5%)	7 (11%)	29 (8%)
Phenotypic resistance to moxifloxacin						
Resistant	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Sensitive	115 (93%)	59 (94%)	55 (93%)	54 (95%)	56 (89%)	339 (93%)
Missing	8 (7%)	4 (6%)	4 (7%)	3 (5%)	7 (11%)	26 (7%)
Phenotypic resistance to ethambutol						
Resistant	0 (0%)	0 (0%)	0 (0%)	1 (2%)	0 (0%)	1 (0%)
Sensitive	112 (91%)	59 (94%)	55 (93%)	53 (93%)	56 (89%)	335 (92%)
Missing	11 (9%)	4 (6%)	4 (7%)	3 (5%)	7 (11%)	29 (8%)
Phenotypic resistance to pyrazinamide						
Resistant	4 (3%)	2 (3%)	2 (3%)	0 (0%)	4 (6%)	12 (3%)
Sensitive	110 (89%)	57 (90%)	53 (90%)	55 (96%)	53 (84%)	328 (90%)
Missing	9 (7%)	4 (6%)	4 (7%)	2 (4%)	6 (10%)	25 (7%)

*An additional three patients were randomised in error and did not start treatment or remain in follow-up and are therefore not included in this table. $RIF_{35}HZE$=rifampicin 35 mg/kg, isoniazid, pyrazinamide, ethambutol. RIFQHZ =rifampicin 10 mg/kg, isoniazid, pyrazinamide, SQ109 300 mg. $RIF_{20}QHZ$=rifampicin 20 mg/kg, isoniazid, pyrazinamide, SQ109 300 mg. $RIF_{20}MHZ$=rifampicin 20 mg/kg, isoniazid, pyrazinamide, moxifloxacin 400mg. Data are median (IQR) or n (%) unless otherwise specified. Doses of concomitant drugs are detailed in Procedures.

***Table 1*: Baseline characteristics by arm**

Procedures

All drugs were oral, given once daily, 7 days per week. Control treatment consisted of standard dose rifampicin (10 mg/kg [range 8·1–11·8 mg/kg]), isoniazid (5 mg/kg [4·1–5·9 mg/kg]), pyrazinamide (25 mg/kg [21·6–31·6 mg/kg]), and ethambutol (15–20 mg/kg [14·9–21·7 mg/kg]) for 8 weeks, followed by 18 weeks of standard dose rifampicin and isoniazid. Experimental treatments consisted of rifampicin 35 mg/kg, plus standard dose isoniazid, pyrazinamide, and ethambutol (arm $RIF_{35}HZE$); rifampicin 10 mg/kg, standard dose isoniazid and pyrazinamide, and SQ109 300 mg (arm RIFQHZ); rifampicin 20 mg/kg, standard dose isoniazid and pyrazinamide, and SQ109 300 mg (arm $RIF_{20}QHZ$); or rifampicin 20 mg/kg, standard dose isoniazid and pyrazinamide, and moxifloxacin 400 mg (arm $RIF_{20}MHZ$). Experimental treatment was given for 12 weeks, followed by 14 weeks of standard dose isoniazid and rifampicin. Pyridoxine (vitamin B6, 25 mg) was given in the morning to all patients throughout the trial. The control regimen was weight banded according to the South African tuberculosis treatment guidelines[18] and implemented in both participating countries (appendix p 10). Higher doses of rifampicin were adapted to these weight bands (appendix). All drugs were self-administered except for days of clinic visits, where administration was directly observed. Participants were advised to take their drugs in the morning after a light breakfast and with a glass of water.

The primary study objective was to assess whether the experimental regimens, given for 12 weeks, resulted in shorter time to sputum culture conversion in liquid media compared with standard treatment. Patients were seen once per week up to week 12, and at weeks 14, 17, 22, and 26 after start of treatment. Sputum for smear and culture was taken 2 days before start of treatment, and at all visits. Samples were processed and cultured in liquid broth medium culture according to the mycobacteria growth indicator tube (Bactec MGIT960) system, on Löwenstein-Jensen (LJ) solid medium; and sensitivity to isoniazid, rifampicin, and ethambutol was assessed by liquid culture susceptibility testing (SIRE) at baseline and positive cultures after week 12, following the procedures described previously.[15] Safety assessments included physical examination and vital signs. Liver function tests, lipase, electrolytes, glucose, and haematology were done at screening and treatment weeks 1, 2, 4, 6, 9, 12, and 14. Coagulation assessments were done at screening and at week 2, and ECGs at screening, baseline, and at week 1 and 2. The Friderica formula was used for heart rate correction of QT.[19] All laboratory staff and ECG analysts were blinded to treatment allocation. Post-treatment follow-up was introduced during

enrolment, following the IDMC review of the results of the first interim analysis. Follow-up was done at 3 months and 6 months after end of treatment by telephone, and patients were invited to attend the clinic for assessment if feeling unwell.

An electronic source documentation system (Clinical Ink, Winston Salem, NC), was used for clinical and laboratory data capture. All data were stored in a central study database to facilitate regular monitoring of data quality and completeness.

Outcomes

The primary endpoint was time from treatment initiation to the first of two consecutive negative once-weekly sputum cultures without an intervening positive culture in liquid media, up to 12 weeks. Secondary endpoints were time to first negative culture in liquid and solid media, the proportion of patients converting to negative sputum culture in liquid and solid media at each time-point during treatment, rate of change in time to positivity in liquid culture and frequency of treatment failures or development of drug resistance in the different experimental arms, and safety. Modelling of rate of change in time to positivity in liquid culture is ongoing and will be reported more fully in a subsequent modelling paper.

Mycobacteriology endpoints were acquisition of resistance against rifampicin, pyrazinamide, isoniazid, ethambutol, and moxifloxacin during therapy. Minimum inhibitory concentrations and their change over treatment, and strain typing by genome sequencing that allows true relapse to be distinguished from reinfection are other endpoints that are being assessed and will be reported in a subsequent paper.

Adverse events were registered according to the Common Terminology Criteria for Adverse Events 4.03 (CTCAE).[20] An adverse event was classified as serious if it led to death, permanent or significant disability, was a congenital anomaly or birth defect, was life threatening, or required hospital admission for management. Safety and efficacy results were reviewed by the IDMC at 6-monthly intervals during the trial. A trial steering committee with independent chair and majority supervised the conduct of the trial and made decisions following recommendations from the IDMC.

Pharmacokinetics sub-study

20 patients allocated to each study arm, divided equally between the South African and Tanzanian sites, were enrolled into a pharmacokinetics sub-study where blood was taken at treatment week 4. A full description of the pharmacokinetic sub-study is provided in the appendix (pp 13–15).

Statistical analysis

We assumed culture conversion of 85% by 12 weeks in the control arm[14] and a 5% loss to follow-up, which meant that 124 patients in the control arm and 62 in the experimental arm would be adequate to detect a hazard ratio of 1·8 with 90% power and a two-sided type I error of 5% for each pairwise comparison (a total of 372 patients). We selected a hazard ratio (HR) of 1·8 as a criterion to indicate potential for treatment shortening, since this represented an increase over the 1·68 HR obtained in the moxifloxacin-containing phase 2 trial that led to the phase 3 REMoxTB trial.[14,18]

Up to two interim IDMC analyses were planned after 28 and then 50 patients from the control arm had

	Control	$RIF_{35}HZE$	RIFQHZ	$RIF_{20}QHZ$	$RIF_{20}MHZ$	Total
Total in analysis (mITT)	123	63	58	56	63	363
Number of culture conversions during 26-week follow-up (MGIT culture)	101 (82%)	51 (81%)	44 (76%)	48 (86%)	52 (83%)	296 (82%)
Number of culture conversions during 26-week follow-up (solid culture)	117 (95%)	59 (94%)	59 (97%)	54 (96%)	59 (94%)	345 (95%)
Primary analysis to 12 weeks (MGIT culture)						
Cumulative probability of culture conversion by 12 weeks	70·1%	79·9%	65·2%	58·6%	78·7%	..
Median time to culture conversion (IQR)	62 (41–83)	48 (34–69)	63 (48–83)	66 (41–83)	55 (41–69)	..
Adjusted hazard ratio (95%)*	..	1·78 (1·22–2·58) p=0·003	0·85 (0·57–1·27) p=0·42	0·76 (0·50–1·17) p=0·21	1·42 (0·98–2·05) p=0·07	..
Hazard ratio (95%), unadjusted	..	1·46 (1·02–2·11) p=0·04	0·90 (0·60–1·34) p=0·60	0·76 (0·50–1·16) p=0·21	1·34 (0·93–1·93) p=0·12	..
Solid LJ culture to 12 weeks (secondary)						
Cumulative probability of culture conversion by 12 weeks	97·3%	100·0%	94·4%	94·2%	98·0%	..
Median time to culture conversion (IQR)	27 (13–48)	20 (7–41)	20 (7–48)	20 (11–44)	29 (20–48)	..
Adjusted hazard ratio (95% CI)*	..	1·23 (0·89–1·69) p=0·21	0·91 (0·66–1·27) p=0·58	0·98 (0·70–1·38) p=0·93	0·77 (0·56–1·06) p=0·11	..
Unadjusted hazard ratio (95% CI)	..	1·28 (0·93–1·75) p=0·13	1·02 (0·73–1·41) p=0·92	1·06 (0·76–1·47) p=0·74	0·90 (0·65–1·23) p=0·50	..

LJ=Löwenstein-Jensen. MGIT=mycobacteria growth indicator tube. mITT=modified intention to treat. $RIF_{35}HZE$=rifampicin 35 mg/kg, isoniazid, pyrazinamide, ethambutol. RIFQHZ=rifampicin 10 mg/kg, isoniazid, pyrazinamide, SQ109 300 mg. $RIF_{20}QHZ$=rifampicin 20 mg/kg, isoniazid, pyrazinamide, SQ109 300 mg. $RIF_{20}MHZ$=rifampicin 20 mg/kg, isoniazid, pyrazinamide, moxifloxacin 400 mg. Doses of concomitant drugs are detailed in Procedures. *Analysis adjusted for HIV status, GeneXpert cycle threshold (<16, ≥16), and site. MGIT analyses also adjusted for baseline time to positivity.

***Table 2*: Summary of analyses of time to culture conversion in MGIT culture (primary) and on solid LJ culture (secondary) to 12 weeks**

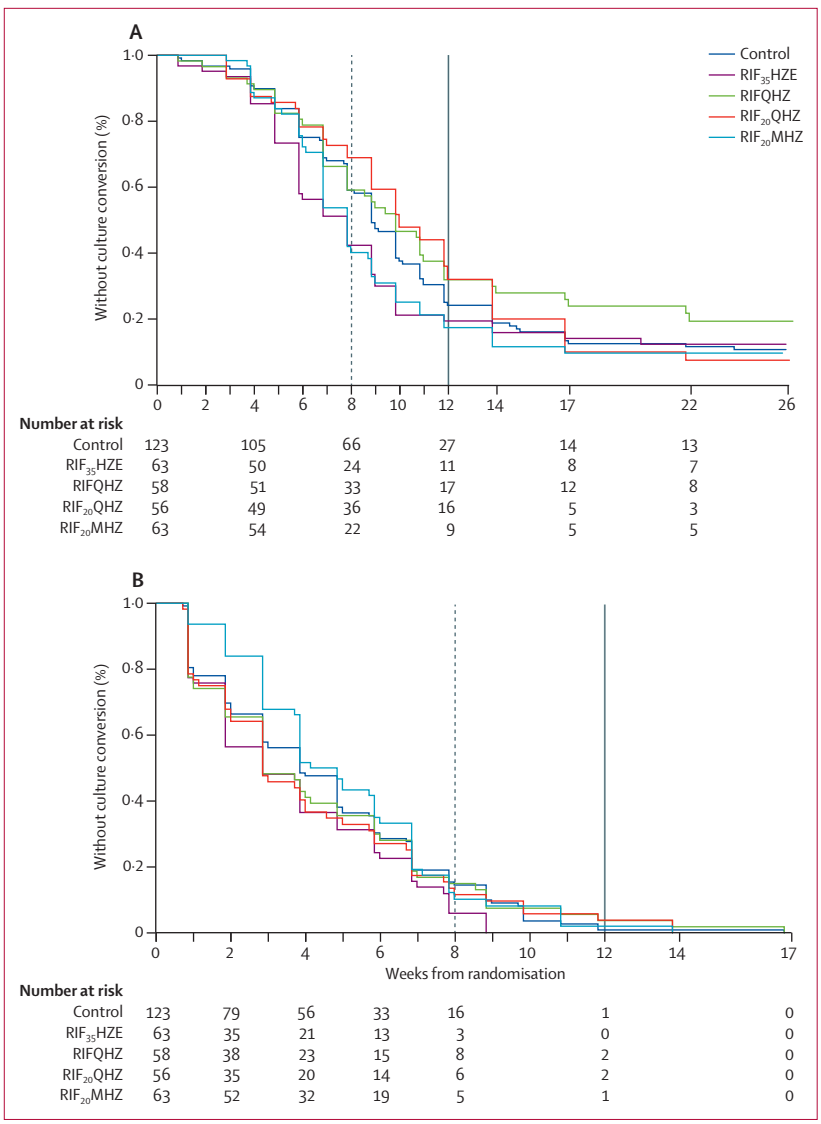

***Figure 2*: Kaplan-Meier curve for time to culture conversion**
(A) Time to culture conversion in liquid MGIT media. (B) Time to culture conversion on solid Löwenstein-Jensen media. MGIT=mycobacteria growth indicator tube. $RIF_{35}HZE$=rifampicin 35 mg/kg, isoniazid, pyrazinamide, ethambutol. RIFQHZ=rifampicin 10 mg/kg, isoniazid, pyrazinamide, SQ109 300 mg. $RIF_{20}QHZ$=rifampicin 20 mg/kg, isoniazid, pyrazinamide, SQ109 300 mg. $RIF_{20}MHZ$=rifampicin 20 mg/kg, isoniazid, pyrazinamide, moxifloxacin 400 mg. Doses of concomitant drugs are detailed in Procedures. Dashed vertical line refers to the week 8 time-point (cutoff in post-hoc analysis). Solid vertical line refers to the week 12 time-point (cutoff in primary analysis).

achieved stable culture conversion endpoint. Recruitment was to be terminated in experimental arms with HR less than 1·09 (first interim) or less than 1·23 (second interim). The probability of continuing an efficacious arm at each interim analysis—the stage-wise power—was 0·95, and the probability of not dropping an inefficacious arm—the stage-wise type I error—was 0·4 (first interim) and 0·2 (second interim).

Patients without evidence of rifampicin resistance on phenotypic test who took at least one dose of study treatment and had one positive culture on liquid or solid media before or within the first 2 weeks of treatment were included in the primary analysis population (a modified intention-to-treat [ITT] population). In secondary analyses, the primary endpoint was also analysed on an ITT population (all randomly assigned patients) and a per-protocol population (PP, the modified ITT population, excluding randomly assigned patients that did not meet the eligibility criteria and patients that missed 21 or more doses of their allocated treatment in the first 12 weeks). The modified ITT population was not included in the original trial protocol, but was included as the primary analysis population in the statistical analysis plan before any interim analyses to allow comparison with other tuberculosis trials. Including patients without culture-confirmed disease (ITT) or excluding patients based on post-randomisation data (PP) can introduce bias, and therefore the modified ITT population was preferred. Time-to-event data were analysed using a Cox proportional-hazards regression model and adjusted for minimisation variables (HIV status, Xpert MTB/RIF cycle threshold, centre) and for the baseline liquid culture bacterial load measurement, time to positivity (mean of pre-treatment cultures) for analyses of liquid culture data. Log-rank statistics were also calculated for time-to-event data, both unstratified and stratified by minimisation variables. The proportional-hazards assumption was tested using Schoelfeld residuals, with $p<0·05$ evidence for non-proportionality. Details of secondary outcomes and sensitivity analyses are described in the appendix. Post-hoc analyses for time to stable culture conversion to 8 weeks on liquid and solid media that were not prespecified in the statistical analysis plan were done for comparability with previous phase 2 studies.

To assess safety, the proportion of adverse events and the proportion of adverse events assessed by the investigators as probably related or related to treatment was presented by group. Data were analysed using Stata version 13.1 (StataCorp, College Station, Texas).

Role of the funding source

The funders of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication. Sequella, Inc provided SQ109. Sequella had no role in study design, data collection, data analysis, and data interpretation; they reviewed the paper and made comments.

Results

Between May 7, 2013, and March 25, 2014, 632 patients were screened and 365 were enrolled in Tanzania (51 in Moshi, 52 in Mbeya, and 53 in Bagamoyo) and South Africa (88 in Cape Town, 48 in Stellenbosch, 30 in Johannesburg, and 43 in Tembisa). Three additional patients were randomised in error and were not started

	Control	$RIF_{35}HZE$	RIFQHZ	$RIF_{20}QHZ$	$RIF_{20}MHZ$
MGIT culture censored at 8 weeks (post hoc)					
Cumulative probability of culture conversion by 8 weeks	32%	49%	34·5%	27·8%	46·2%
Adjusted hazard ratio (95% CI)*	..	2·06 (1·26–3·38) p=0·004	1·04 (0·59–1·81) p=0·90	0·91 (0·49–1·67) p=0·76	1·67 (1·01–2·67) p=0·05
Unadjusted hazard ratio (95% CI)	..	1·73 (1·07–2·82) p=0·03	1·07 (0·62–1·86) p=0·81	0·87 (0·48–1·58) p=0·64	1·47 (0·90–2·40) p=0·13
Solid LJ culture censored at 8 weeks (post hoc)					
Cumulative probability of culture conversion by 8 weeks	80·9%	88·0%	83·9%	82·6%	82·7%
Adjusted hazard ratio (95% CI)*	..	1·17 (0·83–1·64)	1·00 (0·70–1·42)	1·06 (0·74–1·52)	0·76 (0·54–1·07)
Adjusted log-rank test*	..	p=0·38	p=1·00	p=0·75	p=0·12
Unadjusted hazard ratio (95% CI)	..	1·24 (0·88–1·73)	1·09 (0·77–1·55)	1·12 (0·79–1·60)	0·88 (0·63–1·24)
Unadjusted log-rank test	..	p=0·22	p=0·62	p=0·53	p=0·48
Solid LJ culture censored at 12 weeks excluding without a positive culture on LJ solid media before or within the 2 weeks of randomisation (post hoc)					
Number in analysis (total=297)	101	46	45	47	58
Cumulative probability of culture conversion by 8 weeks	96·7%	100·0%	92·8%	93·3%	97·8%
Adjusted hazard ratio (95% CI)*	..	1·37 (0·95–1·99)	0·84 (0·58–1·23)	1·00 (0·69–1·45)	0·88 (0·62–1·24)
Adjusted log-rank test*	..	p=0·19	p=0·78	p=0·62	p=0·37
Unadjusted hazard ratio (95% CI)	..	1·37 (0·95–1·98)	0·92 (0·64–1·34)	1·05 (0·73–1·51)	0·95 (0·67–1·33)
Log-rank test, unadjusted	..	p=0·07	p=0·65	p=0·76	p=0·73

LJ=Löwenstein-Jensen. MGIT=mycobacteria growth indicator tube. $RIF_{35}HZE$=rifampicin 35 mg/kg, isoniazid, pyrazinamide, ethambutol. RIFQHZ=rifampicin 10 mg/kg, isoniazid, pyrazinamide, SQ109 300 mg. $RIF_{20}QHZ$=rifampicin 20 mg/kg, isoniazid, pyrazinamide, SQ109 300 mg. $RIF_{20}MHZ$=rifampicin 20 mg/kg, isoniazid, pyrazinamide, moxifloxacin 400 mg. Doses of concomitant drugs are detailed in Procedures. *Analysis adjusted for HIV status, GeneXpert cycle threshold (<16, ≥16), and site. MGIT analyses also adjusted for baseline time to positivity.

***Table 3*: Summary of analyses of time to culture conversion in MGIT and on LJ culture to 8 weeks (post hoc), and on LJ culture excluding patients without positive LJ at baseline (post hoc)**

on treatment and not continued in follow-up (figure 1). Recruitment to the RIFQHZ and $RIF_{20}QHZ$ arms was terminated following the recommendation from the IDMC after review of results from the first scheduled interim analysis; however, enrolled patients continued on allocated treatment. Recruitment to all other arms continued until the target sample sizes in each arm had been reached. Recruitment was completed before the scheduled second interim analysis, which was therefore not done.

Baseline characteristics were similar between arms (table 1). Two patients were rifampicin resistant on phenotypic tests and were excluded from the modified ITT analysis population.

Patients achieved faster stable culture conversion in MGIT on the $RIF_{35}HZE$ arm than on the control arm, with a median time to stable culture conversion of 48 days compared with 62 days (adjusted HR 1·78, 95% CI 1·22–2·58; p=0·003, table 2, figure 2). HR was smaller in the unadjusted analysis (HR 1·46, 95% CI 1·02–2·11; p=0·04). None of the other arms reported significantly faster culture conversion than control, with the largest effect seen in the $RIF_{20}MHZ$ arm (adjusted HR 1·42, 95% CI 0·98–2·05; p=0·07). The cumulative proportion of patients achieving the primary endpoint of culture conversion in liquid media at week 12 (using the Kaplan-Meier estimator) was 70·1% (control), 79·9% ($RIF_{35}HZE$), 65·2% (RIFQHZ), 58·6% ($RIF_{20}QHZ$), and 78·7% ($RIF_{20}MHZ$).

Time to culture conversion on solid media did not differ significantly between any of the arms and the control arm. In the post-hoc analysis for comparability with previous tuberculosis phase 2 trials[14,21] at 8 weeks, HR for the primary endpoint on liquid culture was higher for $RIF_{35}HZE$ (adjusted HR 2·06, 95% CI 1·26–3·38; p=0·004) and for $RIF_{20}MHZ$ (1·67, 1·01–2·67; p=0·05, table 3). None of the arms reported significantly faster time to culture conversion than the control arm on solid media when data were censored at 8 weeks. To understand the differences in results between solid and liquid media, we did a further post-hoc analysis of time to culture conversion by excluding patients without a positive culture on solid media before or within the 2 weeks of randomisation; this analysis gave HRs that were marginally closer than those in liquid media, but differences between solid and liquid media remained (table 3). Results of further sensitivity analysis for the primary outcome and the secondary outcomes time to first negative culture in liquid and solid media and proportion of patients converting to negative sputum culture in liquid and solid media at each timepoint during treatment are presented in the appendix (pp 16–18).

For an **interactive graph on sensitivity analysis** see http://www.ctu.mrc.ac.uk/resources/panacea/mamstb/

	Control	$RIF_{35}HZE$	RIFQHZ	$RIF_{20}QHZ$	$RIF_{20}MHZ$	Total
Total in safety analysis	123	63	59	57	63	365
Patients with at least one AE	92 (75%)	53 (84%)	49 (83%)	42 (74%)	49 (78%)	285 (78%)
Patients with at least one grade 3, 4, or 5 AE	13 (11%)	9 (14%)	7 (12%)	7 (12%)	9 (14%)	45 (12%)
Patients with at least one grade 3, 4, or 5 AE considered probably related or related	1 (1%)	3 (5%)	0	0	4 (6%)	8 (2%)
Patients with at least one serious AE	6 (5%)	4 (6%)	4 (7%)	5 (9%)	4 (6%)	23 (6%)
Deaths	0	1	0	0	0	1
Total number of patients with treatment changed due to hepatic AE	2 (2%)	5 (8%)	0 (0%)	3 (5%)	0	10 (3%)
Number of patients with treatment changed due to hepatic AE—symptomatic or meeting protocol criteria*	2 (2%)	3 (5%)	0 (0%)	3 (5%)	0	8 (2%)
Treatment changed due to hepatic AE—not fulfilling protocol criteria and not being symptomatic*	0	2 (3%)	0	0	0	(1%)

AE=adverse event. AST=aspartate aminotransferase. ALT=alanine transaminase. $RIF_{35}HZE$=rifampicin 35 mg/kg, isoniazid, pyrazinamide, ethambutol. RIFQHZ=rifampicin 10 mg/kg, isoniazid, pyrazinamide, SQ109 300 mg. $RIF_{20}QHZ$=rifampicin 20 mg/kg, isoniazid, pyrazinamide, SQ109 300 mg. $RIF_{20}MHZ$=rifampicin 20 mg/kg, isoniazid, pyrazinamide, moxifloxacin 400 mg. Doses of concomitant drugs are detailed in Procedures. *Protocol criteria for treatment interruption due to hepatic AE: elevation of AST or ALT more than three times, but less than five times the upper limit of normal, with associated symptoms or elevation of AST or ALT more than five times the upper limit of normal irrespective of the presence of symptoms.

Table 4: **Summary of adverse events**

One patient with baseline isoniazid mono-resistance treated with RIFQHZ acquired rifampicin and pyrazinamide resistance and was classified as treatment failure at week 17. The patient received appropriate treatment for multi-drug resistant tuberculosis and was reported cured after follow-up. Acquired drug resistance was not seen in any other patients. Seven patients were diagnosed with recurrent disease at 12 months after randomisation, and three more were identified by non-study health facilities without culture confirmation (one by smear and two by Xpert MTB/RIF; figure 1). Of these 11 unfavourable outcomes, only one occurred on the $RIF_{35}HZE$ arm and none on the $RIF_{20}MHZ$ arm, and all had longer time to conversion to negative culture in liquid media (median 97 days *vs* 62 days in those without failure or recurrence, p=0·013), but not on solid media (median 27 days *vs* 27 days, p=0·157).

45 patients reported at least one grade 3, 4, or 5 adverse event with similar proportions in each arm (table 4). There was one death during the trial, a patient on the $RIF_{35}HZE$ arm who died 13 weeks after experimental treatment was completed, near the end of the continuation phase, and who complained of sudden onset chest pain hours before death. This death was unlikely to be related to the higher dose of rifampicin. One patient in the control arm completed treatment successfully but later relapsed and died from underlying pneumoconiosis, related to work as a miner in Tanzania. Eight patients with hepatic adverse events fulfilled the criteria for treatment interruption (table 4). Investigators opted for treatment interruption in two additional cases of grade 2 elevated transaminases in arm $RIF_{35}HZE$, in whom the protocol toxicity criteria for interruption were not fulfilled. Five patients were restarted on study treatment, which was tolerated by three. No QTcF interval of more than 480 ms was recorded during the study. Further details of adverse events are in the appendix (p 12).

Increasing the rifampicin dose from 10 to 35 mg/kg resulted in a more than proportional increase in the geometric mean area under the curve $(AUC)_{0-24h}$ and maximum concentration (C_{max}) of rifampicin [appendix, pp 12–15, 21]. No significant differences in isoniazid, pyrazinamide, or ethambutol exposure were found between standard and higher dose rifampicin arms, indicating that higher doses of rifampicin do not affect the exposures to these drugs. Tanzanians had a lower rifampicin AUC_{0-24h} than South Africans in the $RIF_{35}HZE$ arm (geometric means 145 and 206 h×mg/L, 11 *vs* 9 patients, p=0·001). Pyrazinamide AUC_{0-24h} values were also lower in Tanzanians than South Africans (317 and 360 h×mg/L, 94 patients, p=0·02). Plasma concentrations of SQ109 in the study are not yet available, a pharmacokinetic-pharmacodynamic analysis is ongoing, and will be reported separately.

Discussion

Our study shows that a regimen including rifampicin 35 mg/kg resulted in significantly faster liquid culture conversion by 12 weeks. Previous tuberculosis trials have used culture conversion at 8 weeks, and the 8-week adjusted HR of 2·06 in our trial is the highest reported for any tuberculosis regimen; HRs from other trials include 1·68 for pretomanid,[21] 1·52 for gatifloxacin,[14] and 1·68 for moxifloxacin.[14] Only a study[22] of high dose rifapentine reported similar activity in terms of increasing the proportion of patients with sputum culture conversion at 8 weeks. Culture conversion rates in our study at weeks 12 and 26 were lower than in comparable trials. One patient had treatment failure with persistent positive cultures, but the others did not achieve the endpoint because of the stringent definition used,

which requires two negative cultures from successive visits, and is susceptible to missing culture results by contamination or missing sample.

None of the other arms in our study resulted in faster culture conversion in liquid or on solid culture compared with control. Although $RIF_{20}MHZ$ was the best of 20 mg/kg rifampicin regimens, it was not better than moxifloxacin containing regimens with standard dose rifampicin reported previously.[14,15] This could be due to relatively low exposure to moxifloxacin in our cohort, considering that moxifloxacin is metabolised by the phase 2 metabolising enzymes uridine diphosphate glucuronosyltransferase and sulphotransferase. Rifampicin is known to induce such phase 2 metabolic enzymes and it has been shown that rifampicin decreases the AUC of moxifloxacin by approximately 30%.[23] The results in the $RIF_{20}MHZ$ arm suggest that 20 mg/kg rifampicin does not result in enhanced efficacy. SQ109 did not reach the prespecified HR in the dose and combinations used. This drug is metabolised by cytochrome P450 iso-enzymes CYP2D6 and CYP2C19, and at least CYP2C19 can be induced by rifampicin. We have previously shown that rifampicin decreases exposure to SQ109 administered in a 150 mg daily dose, but a higher daily dose of 300 mg SQ109, as used in our study, outweighed this inductive effect of rifampicin.[13]

Clinical outcome was assessed at 12 months post randomisation (6 months after completion of treatment) and recurrence rates were similar between regimens.

Faster conversion in the $RIF_{35}HZE$ arm was only seen with liquid but not with solid culture, unlike a large trial assessing moxifloxacin regimens where the culture conversion results were the same on liquid and solid media.[15] Rifampicin is considered to be effective not only on actively replicating but also on persisting, non-replicating bacterial phenotypes that are drug tolerant.[24] Incomplete killing of such bacilli is one of the proposed reasons for relapse after treatment completion[25] as is poor penetration to the site of infection.[26] Higher doses of rifampicin seem to more effectively kill this sub-population in experimental models[4,24] and in human beings.[27,28] Since liquid media, unlike solid media, detect such bacilli,[29] we believe that the higher efficacy of $RIF_{35}HZE$ seen in liquid media represents increased activity of this regimen on this bacterial phenotype. The relevance of the liquid media endpoint is further supported by the fact that patients with treatment failure or recurrent disease had later culture conversion on liquid media, but not on solid media (although numbers were small).

Increasing the dose of rifampicin from 10 to 35 mg/kg resulted in a more than proportional increase in exposure to rifampicin, which is in agreement with previous data[30] and with more recent pharmacokinetic results from our rifampicin dose ranging trial.[8] The response associated with higher doses and exposures to rifampicin is also in agreement with in-vitro, infected macrophage, and in-vivo murine data that showed an increased bactericidal effect and shortening of treatment duration once the steep part of the rifampicin dose–exposure-response curve is reached.[3,6] In this respect, the 35 mg/kg rifampicin regimen is promising but might not yet be optimal. Our ongoing phase 2a dose escalating study indicates that a higher dose of 40 mg/kg is also safe and well tolerated.[8,9] The rifampicin dose might yet be increased safely upon the human dose–exposure-response curve, which could aid treatment shortening and reduce the risk of rifampicin resistance for individuals with lower drug exposures.[31] Our data bring into question the utility of testing regimens with only 20 mg/kg rifampicin,[9] suggesting that they are not likely to provide significant benefit or treatment shortening. A trial[32] from Vietnam in tuberculosis meningitis did not find a reduction in mortality with a regimen containing 15 mg/kg oral rifampicin.[32] A higher dose and exposure to rifampicin would also lessen the relevance of the wide inter-individual variability in exposure to rifampicin as observed in this study. Higher AUCs were observed in South Africa than in Tanzania in the 35 mg/kg arm, possibly associated with inter-ethnic differences in genetic polymorphisms of genes encoding for drug transporters or enzymes involved in the metabolism of rifampicin.[33]

The advantage of the concept of high dose rifampicin is that the drug is widely available at low cost and implementation could take place broadly and quickly. A larger pill burden is a potential disadvantage, as available rifampicin formulations are not adapted to the higher doses. After defining the optimal dose of rifampicin new formulations would need to be developed in fixed dose combinations with companion drugs.

Experimental arms had similar safety profiles to the control arm. Treatment interruptions due to hepatic events were few. Although more interruptions occurred in the $RIF_{35}HZE$ arm, in two instances the severity was less than specified by the protocol, and since clinicians were not blinded to treatment allocation this might have influenced the decision to stop treatment.

Our study was, to our knowledge, the first multi-arm, multi-stage trial in infectious diseases, and showed that this design is feasible in a multicentre African setting, and can be used to assess many novel combinations. We identified a regimen that achieved our target and has promise to progress to phase 3, while discontinuing two regimens that did not show sufficient efficacy, thereby saving on resources.

This study has some limitations. It was not powered to be able to differentiate between the experimental regimens, only between individual arms and the control regimen. The sample size to achieve such a power would be inappropriately large. Furthermore, it was not powered for assessment of relapse, which is the definitive endpoint of a

pivotal trial but for time to culture conversion, which is an intermediate endpoint. Our newly developed phase 2c STEP trial design will address this issue in future trials,[34] and will improve confidence in a decision to move an arm into phase 3 based on a relapse endpoint.

Also, our objective was to identify regimens for future phase 3 assessment and thus our study does not provide information on the activity of individual experimental components. The study was only done in Africa and the HIV co-infected population was small. Therefore, future phase 3 trials should include sites from outside Africa and recruit representative proportions of HIV co-infected patients on antiretroviral treatment.

Another limitation is that because of the time required from sputum collection to determine negative cultures (which was necessary for our primary endpoint) and due to faster than expected recruitment, the interim analysis occurred late in the recruitment period. As a result, the multi-arm, multi-stage design resulted in a reduction in the final sample size of only 2%. A real-time biomarker such as the molecular bacterial load assay could overcome this problem by replacing culture allowing the endpoint results to be available rapidly.[35] Nevertheless, this was a crucial opportunity to assess the feasibility of this novel design in the context of an African multicentre infectious diseases clinical trial. In the future, larger trials with more arms, and higher patient numbers per arm will achieve more substantial cost savings through a multi-arm, multi-stage design. These would also produce smaller confidence intervals, allowing a more precise estimate of the true HR. The collection of relapse data piloted in this study has been incorporated into our newly developed phase 2c STEP trial design.[34] Such developments will provide the data to support the decision to move an arm into phase 3.

We have shown that 35 mg/kg rifampicin is safe and reduces time to culture conversion in liquid media. Importantly, the multi-arm, multi-stage concept has been shown to be feasible in a multicentre tuberculosis high burden setting, generating evidence to make a decision for whether or not to proceed to phase 3, and could greatly speed regimen development at reduced cost. In this phase 3 trial, a regimen with high dose rifampicin can be tested for safety and tolerability, potential to shorten duration of treatment, and ability to prevent the emergence of resistance to rifampicin.

Contributors

MJB, NH, RA, SHG, PPJP, and MHo conceived the study, AHD, RD, GSK, GC, IS, NEN, LTM, SC, MHa, HHS, SGM, CM, BM, KR, AV, and KN acted as clinical site investigators and were responsible for acquisition and quality of the data. MJB, NH, RA, SR, AM, SH, AC, GPvB, PPJP, and MHo executed investigator initiated sponsor duties and oversight. RDH was the overall laboratory coordinator. SR, RA, MHo, RSW, and PPJP did the analysis. All authors were involved in interpretation of the data and participated in writing of the manuscript. MJB and NH wrote the first and the final draft.

Declaration of interests

We declare no competing interests.

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Publikation 2:

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High seroprevalence for typhus group rickettsiae, southwestern Tanzania.

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High Seroprevalence for Typhus Group Rickettsiae, Southwestern **Tanzania**

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Rickettsioses caused by typhus group rickettsiae have been reported in various African regions. We conducted a cross-sectional survey of 1,227 participants from 9 different sites in the Mbeya region, Tanzania; overall seroprevalence of typhus group rickettsiae was 9.3%. Risk factors identified in multivariable analysis included low vegetation density and highway proximity.

Murine, or endemic, typhus is primarily caused by *Rickettsia typhi* (typhus group rickettsiae [TGR]) and is usually manifest as a benign disease. A systemic vasculitis causes a clinical triad of fever, headache, and maculopapular rash (*1*). Because these signs and symptoms are nonspecific, the disease is often misdiagnosed or overlooked and can frequently be misclassified as malaria (*2*,*3*). In rare cases, murine typhus can lead to severe systemic complications such as acute renal failure, interstitial pneumonia, and complications of the central nervous system. The case-fatality-rate is <5% (*2*), in contrast to the situation for epidemic, or louse-borne, typhus caused by *R. prowazekii*, which can produce severe disease and fatality rates up to 30% if untreated. Serologic tests cannot distinguish these 2 infections, however. We assume that the antibodies detected in Tanzania in this study were caused by *R. typhi*, because, to our knowledge, no severe or epidemic illness compatible with louse-borne typhus has been described in the study region.

Murine typhus is found throughout the world, widely distributed in subtropical and tropical regions, and is most apparent in port cities with large rat populations (*2*,*4*), which provide a reservoir for the pathogen and its main vector, the rat flea (*Xenopsylla cheopsis*). Additional transmission cycles have been described in Texas and California, USA, which involved mainly suburban cats and opossums as reservoir hosts and the cat flea (*Ctenocephalides felis*) as vector (*5*). Other yet unknown cycles may exist.

In Tanzania, information on typhus is sparse. A seroprevalence study among pregnant women from the port city of Dar es Salaam found a seropositivity prevalence of 28% (*4*). In the landlocked northern Tanzanian town of Moshi, murine typhus was detected in 0.5% of febrile patients (*6*).

A predictive risk model for endemic typhus based on environmental conditions has not been established, but because plague is also transmitted by *X. cheopsis* fleas, some of the findings regarding plague transmission might also apply to murine typhus. However, no data are available on the vector flea *C. felis*, the predominant flea harvested from rodents in a study in Uganda (*7*).

The Study

We conducted a cross-sectional seroprevalence study among 1,227 persons from the Mbeya region in southwestern Tanzania to estimate TGR seroprevalence rates and to assess associated sociodemographic and environmental risk factors. This study was conducted as a substudy within the EMINI (Evaluation and Monitoring the Impact of New Interventions) longitudinal cohort study. Briefly, in 2005 we conducted a census at 9 study sites (Figure) to collect baseline data, and 10% of census households were chosen by geographically stratified random selection to participate in the 5-year EMINI longitudinal cohort study (http://www.mmrp.org/projects/cohort-studies/emini.html). From these, serum specimens for this substudy were selected by stratified disproportionate random sampling of stored samples from 17,872 persons who took part in the second EMINI survey in 2007. Stratification was done for age (7 categories), altitude of residence (2 categories), and ownership of domestic mammals (2 categories) and resulted in 28 strata of roughly similar size, described in detail elsewhere (*8*). Serum samples were tested for IgG against *R. typhi* by indirect immunofluorescence assay (IIFA) with the same batch of a commercially available test (Rickettsia typhi Spot IF; Fuller Laboratories, Fullerton,

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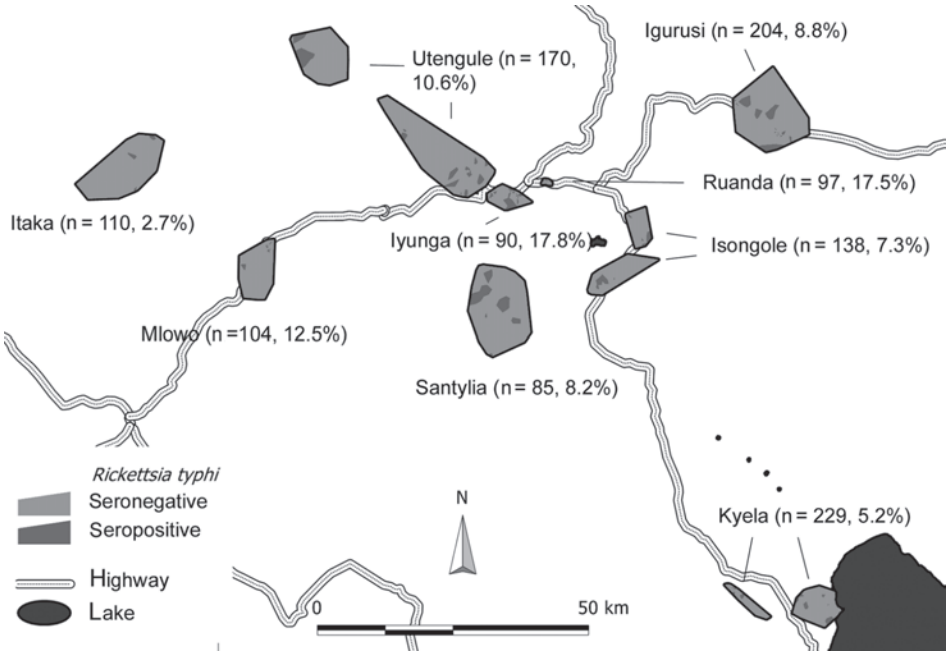

Figure. Study sites in Tanzania, showing positivity and negativity for IgG against *Rickettsia typhi* displayed in Voronoi polygons. Every polygon represents 1 household. Numbers in parentheses indicate site prevalence.

CA, USA.). Samples with an IgG titer of ≥64 or higher were regarded as positive; because IIFA for antibody testing against rickettsiae has a high sensitivity and specificity, as shown by different researchers and with different antigen preparations (*9*). Comparison of the commercial IIFA with a commercial ELISA in our laboratory confirmed this approach (G. Dobler, unpub. data).

To identify possible risk factors for TGR IgG positivity, we analyzed seropositivity as the binary outcome of uni- and multivariable Poisson regression models with robust variance estimates adjusted for household clustering. Initial univariable models for all factors that we deemed as possibly related to TGR infection (Table) were used to identify variables with a univariable p value ≤0.1 for further multivariable evaluation. Stepwise backward and forward regression, the Akaike and Bayesian information criteria, and various assessments of model fit were used to identify the best multivariable model, in which only variables with a multivariable p value <0.1 were retained.

Of the 1,227 analyzed serum specimens, 114 specimens (9.3%) were positive for TGR IgG. This finding translates into an estimated overall population prevalence of 8.4% (95% CI 6.8%–10.1%) when findings are extrapolated from our stratified sample to the underlying population of the 9 sites by using direct standardization. We found local maximum prevalence in the urban sites, Ruanda (17.5%) and Iyunga (17.8%), and in semiurban Mlowo (12.5%; Figure). The prevalence at other sites ranged from 2.7% to 10.6%. The highest seropositivity rate was found in the age quintile from 42.1 to 55.2 years, with a decline thereafter. In univariable analysis, several environmental covariates showed a significant inverse association with TGR IgG (Table), which included vegetation density, rainfall, minimum and night temperatures, whereas population density, cattle density, and socioeconomic status were positively associated with seropositivity. The geographic distribution of seropositive participants (Figure) led us to include distance to the nearest highway as a variable in the analysis. Distance was found to be inversely associated with seropositivity. The final multivariable model included age, vegetation density, and distance to the nearest highway as significant predictors of TGR IgG. Other factors were not included in the multivariable model because their lack of multivariable significance.

Although significant in univariable analysis, the association of population density, rainfall, socioeconomic status, and cattle density became nonsignificant in the multivariable model when vegetation density was included (p = 0.66 for population density; data not shown). Other factors, including sex, livestock ownership, day and night average land surface temperatures, and other environmental factors, were unrelated to TGR seropositivity.

Conclusions

In contrast to results of a recent study of febrile patients from inland northern Tanzania (*6*), site-specific seropositivity prevalences of up to 17.8% in our study suggest that TGR contributes substantially to febrile illness in some areas of the Mbeya region. Our study provides data on environmental risk factors for TGR seropositivity, which might be useful

Table. Covariates associated with seropositivity for typhus group rickettsiae, Mbeya region, southwestern Tanzania, 2007*

Covariate/stratum	No. specimens (% positive)	Univariable†‡ PR (95% CI)	Univariable†‡ p value	Multivariable†§ PR (95% CI)	Multivariable†§ p value
Age, y					
5–<13.6	245 (5.3)	1	–	1 (–)	–
13.6–<27.8	245 (6.5)	1.23 (0.60–2.51)	0.568	1.28 (0.63–2.58)	0.495
27.8–<42.1	243 (12.3)	2.33 (1.23–4.39)	0.009	2.40 (1.28–4.49)	0.006
42.1–55.2	248 (14.9)	2.81 (1.53–5.18)	0.001	2.73 (1.49–4.99)	0.001
>55.2	246 (7.3)	1.38 (0.69–2.76)	0.365	1.41 (0.71–2.80)	0.331
Distance to nearest highway, km	1,227 (9.3)	0.96 (0.94–0.99)	0.012	0.97 (0–0.99)	0.011
Enhanced vegetation index, per 0.1 units	1,227 (9.3)	0.58 (0.44–0.76)	<0.001	0.60 (0.46–0.79)	<0.001
Persons/km^2, per 1,000 persons	1,227 (9.3)	1.08 (1.04–1.12)	<0.001		
Sex					
F	672 (8.6)	1	–		
M	544 (9.9)	1.15 (0.81–1.63)	0.430		
Unknown	11 (18.2)	2.11 (0.59–7.58)	0.254		
SES rank, per unit¶	1,227 (9.3)	1.08 (1.02–1.15)	0.008		
Cattle/km^2, per 100 cattle	1,227 (9.3)	1.28 (1.05–1.56)	0.017		
No. cows owned	1,227 (9.3)	0.96 (0.84–1.09)	0.526		
No. goats owned	1,227 (9.3)	0.94 (0.83–1.07)	0.367		
Dogs owned					
No	820 (8.8)	1	–		
Yes	191 (8.4)	0.95 (0.54–1.67)	0.869		
No information	216 (12.0)	1.37 (0.89–2.11)	0.150		
Minimum ambient temperature, °C	1,227 (9.3)	0.92 (0.87–0.97)	0.004		
Average day land surface temperature, °C	1,227 (9.3)	1.37 (0.71–2.63)	0.351		
Average night land surface temperature, °C	1,227 (9.3)	0.61 (0.38–0.99)	0.044		
Elevation, per 100 m	1,227 (9.3)	1.04 (1.00–1.08)	0.025		
Rainfall, per 1,000 mm	1,227 (9.3)	0.53 (0.30–0.92)	0.025		

*PR, prevalence ratio; SES, socioeconomic status. Blank cells indicate that the variables were not included in the multivariable analyses due to lack of multivariable significance.
†Results of univariable and multivariable Poisson regression adjusted for household clustering by using robust variance estimates.
‡Results of separate models for each of the covariates below.
§Multivariable model, including only age, distance to nearest highway, and enhanced vegetation index as covariates.
¶SES rank, rank (from 0 [lowest] to 10 [highest]), according to socioeconomic score.

to inform a predictive model for disease occurrence. The inverse association of vegetation density with seropositivity has been described for plague in Uganda, a disease that is also transmitted by the rat flea (*X. cheopsis*) (*10*). That study and early laboratory data suggest that dryness is not the driving factor behind the link between vegetation and disease transmission, because increasingly dry conditions in the laboratory adversely affect vector lifespan (*11*). In our study, rainfall was not significantly associated with seropositivity in the multivariable model. The urbanity of a settlement, expressed by population density and closeness to highways, may still be a relevant factor through providing more favorable habitats for the mammal reservoir hosts, and sparse vegetation could just be a proxy for urbanization. The positive univariable association of seropositivity with socioeconomic status appears to be a product of the higher socioeconomic status in urbanized communities. Our results suggest that TGR incidence may increase with deforestation and increasing urbanization. Additional research is needed to detect the pathogen in acute infection and to describe the local transmission cycle to validate the identified risk factors prospectively. We further hypothesize that remote sensing data could be used to design a model for prediction of *R. typhi* infection, which could be used to direct public health interventions in the future.

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ARTICLE

Successful management of an MRSA outbreak in a neonatal intensive care unit

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Abstract We report an MRSA outbreak in our 25-bed tertiary neonatal intensive care unit (NICU), which was successfully contained. Methods include a retrospective review of patient files, microbiology records and meeting protocols. During the seven months of outbreak, 27 patients and seven health care workers (HCWs) had positive cultures for MRSA. The outbreak was caused by the epidemic Rhine-Hessen strain; cultured isolates were monoclonal. After a sharp increase of the number of new MRSA-cases the installation of an outbreak management team (OMT) and implementation of comprehensive measures (extensive screening and decolonization strategy including orally applied vancomycin, isolation wards, intensive disinfection regimen) successfully terminated the outbreak within one month. Ten (53%) of 19 patients with completed follow-up and all of the HCWs were decolonized successfully. Gastrointestinal colonization was present in 15 of 27 (56%) neonates, and was associated with poor decolonization success (30% vs. 78% in absence of gastrointestinal colonization). A comprehensive outbreak management can terminate an outbreak in a NICU setting within a short time. Thorough screening of nares, throat and especially stool is necessary for correct cohorting. Gastrointestinal decolonization in neonates seems difficult.

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Introduction

The immaturity of the immune system, use of broad spectrum antibiotics, surgical procedures, invasive devices and mechanical ventilation expose neonatal intensive care patients to an increased risk of colonization and infection with multidrug-resistant pathogens [1, 2]. Among these, outbreaks caused by Methicillin-resistant *Staphylococcus aureus* (MRSA) are increasingly reported, causing significant morbidity and mortality in neonatal intensive care units (NICUs) [3]. In 2006, Gerber et al. published a consensus paper from the Chicago-Area Neonatal MRSA Working Group on containment and prevention measures, stating that hospitals took substantially different approaches for MRSA control [4]. Prospectively randomized trials comparing different methods of screening and decolonization treatment are lacking in this high risk neonatal population [5, 6].

This article discusses our experiences and conclusions related to the successful containment of an MRSA outbreak

involving 27 neonates and seven health care workers in a German level III NICU.

Materials and methods

Setting and study population

The Department of Neonatology of the University of Bonn is a tertiary neonatal care unit, providing 25 beds in a level III NICU and a neonatal intermediate care unit (NIMC), which are situated at a distance of about 8 km apart. Approximately 400 infants are admitted per year. All neonates admitted to the NICU during the outbreak period were included into this retrospective analysis. Basic clinical patient data of MRSA-colonized infants were extracted from patients' files. Microbiological results were obtained from the database of the hospital laboratory (Institute of Medical Microbiology, Immunology and Parasitology, University of Bonn). All patients with at least one culture positive for MRSA were included. Decontamination cycles were counted, as well as treatment cycles with oral vancomycin and intravenous linezolid. All patients with at least one MRSA-positive stool sample were classified as gastrointestinal carriers ('GI-positive'), whereas children with at least two negative stool samples were classified as 'GI negative'.

Cohorting, isolation, and specific disinfection measures

The outbreak was divided into an "endemic" phase from February to October 2005, with 15 patients diagnosed as MRSA positive during that period, and an "epidemic" phase from November 2005 to January 2006, with 12 patients newly diagnosed in November only, and no patients newly identified thereafter.

During the endemic phase, preventive measures included isolation or barrier nursing of patients, a hygiene training and voluntary screening of health care workers, routine disinfection measures (usual hand disinfection, wiping technique for surfaces), and environmental investigations by contact plate samples from diverse hand-contact and skin-contact surfaces in the patient's immediate environment.

The sharp increase in case number in November prompted more intensive measures. An outbreak management team (OMT) was installed in order to implement a detailed concept for screening, hygiene, decontamination and cohorting. The NIMC ward was exclusively used to cohort all patients with known MRSA colonization ("isolation ward"). All non-colonized children were transferred to the NICU, in which all surfaces had been thoroughly disinfected using the spray-fogging technique with terminal wiping of surfaces. After release of MRSA patients from the NIMC ward, rooms were disinfected in the same fashion. Patients newly admitted to the NICU were isolated in a single room, until their MRSA carrier state was determined.

In addition to hand disinfection, staff and family members were instructed to wear gloves, gowns, and a surgical face mask during any contact to an MRSA-colonized patient or to any potential fomites in the patient's vicinity.

MRSA screening of neonates

Before the outbreak, routine MRSA screening was not practised. Once installed, the OMT implemented screening of nasal, pharyngeal, and rectal swabs and stool specimens sampled 24 h after admission, and then once weekly.

Three days after the completion of each cycle of decolonization treatment, swabs were taken from the nares, throat, axilla, anus, groins, and stool three times on consecutive days to assess decolonization success.

MRSA screening of health care workers

Health care workers were screened by nasal and pharyngeal swabs, mandatory screening was introduced during the epidemic phase.

Microbiology

Microbiological analysis of swabs was performed using MRSASelect agar (Bio-Rad Laboratories, Marnes-la-Coquette, France), a chromogenic medium, which delivers rapid differentiation based on the activity of *mec*A gene product.

Pulsed field gel electrophoresis (PFGE)

Chromosomal DNA for the SmaI (Roche, Germany) restriction digests was purified as described previously [7]. Pulsed-field gel electrophoresis (PFGE) was performed on the Chef DRIII system (Bio-Rad, Germany) using Pulsed Field Certified Agarose (1%) (Bio-Rad), 6 V/cm, a field angle of 120°, a switch time of 5–15 s for 7 h and 15–60 s for a further 19 h. *Staphylococcus aureus* NCTC 8325 served as a standard.

Phage typing

Phage typing was performed with the international phage typing set (IPS) (group I: 29, 52, 52A, 79, 80; group II: 3A, 3 C, 55, 71; group III: 6, 42E, 47, 53, 54, 75, 77, 83A, 84, 85; group V: 94, 96; group M: 81, 95) for *S. aureus* at 100x

routine test dilution. Additionally, the MRSA phage typing set (F30, F33, F38, MR8, MR12, MR25, M3, M5, 622, 56B) and additional regional phages (D11, 19 92, 187 and 192) were employed. Phage types were determined by the strong reactions according to the international rules. Two type strains of the Rhine-Hessen strain (*S. aureus* 3391/02 and 2981/04) were received from the German National Reference Center for Staphylococci, Wernigerode.

Decolonization regimen for patients and HCWs

Total body washing of colonized neonates with diluted polyhexanide (Sanalind®, Fresenius KABI, Bad Homburg, Germany) was done once daily. Mupirocin cream (Turixin®, GlaxoSmithKline, Munich, Germany) was applied to the nares three times per day for five days. In selected patients with persistent gastrointestinal colonization, vancomycin was given orally (30 mg/kg/d in three single doses) for 10 days, together with Lactobazillus GG capsules.

Decolonization of HCWs included nasal mupirocin, throat and skin application of polihexanide and/or octenidine, daily change and washing (>60°C) of clothes and bedding, instruction in hand disinfection techniques, and daily disinfection of hand-contact surfaces (working and home setting).

Statistical analysis

Data were analysed using Epi Info (version 6; CDC, Atlanta, GA) and rates were compared using the chi-square test or Fisher's exact test as appropriate.

Results

The outbreak lasted for 11 months (Feb 2005–Jan 2006) and involved 27 patients. During the following year, no MRSA was detected in any patient in the NICU. Of 358 admissions during the outbreak period, 27 (7.5%) patients had at least one culture positive for MRSA. None of these patients were transferred in from an external hospital. The 27 MRSA-positive patients had a median birth weight of 1,550 g (interquartile range [IQR] 1,073–1,765 g); the median gestational age at birth was 31 weeks (IQR, 29–34 weeks). The median duration of hospitalization was 51 days (IQR, 25.5–83 days). In none of these patients, the length of stay in hospital was prolonged as a result of MRSA colonization or infection.

The index patient was born at 34+0 weeks of gestation. She suffered from congenital generalized lymphangiectasia, and required long-term mechanical ventilation. MRSA was first detected in ascites and pleural effusion during a systemic infection after retransfer from surgery. Intravenous treatment with Linezolid resulted in negative culture results 4 days later. Surveillance cultures remained negative for three weeks; afterwards, MRSA was again cultivated from her nares. In the following course, swabs and ascites cultures remained negative until the patient died from her underlying disease at day 309 of her hospitalization.

In the other colonized patients, no symptoms attributable to MRSA were detected, apart from mild conjunctivitis in one patient.

Neonatal screening results and sampling site

MRSA was most frequently detected in nasal swabs followed by specimens from throat and stool.

Swabs from skin (axilla, groins, anus) did not contribute to detection of persistent colonization. Screening of stool, nares and throat would have detected all 25 episodes of persistent colonization. Screening of nares and throat only would have missed five episodes (19%) of persistent colonization.

To assess the possibility of taking less than three swabs or samples per site for detection of persistent colonisation, the number of swabs positive for MRSA out of three was counted. From 21 episodes with three swabs taken on successive days per sampling site, one of three swabs was positive in 11 episodes, 2 of 3 in four episodes, and 3 of 3 in six episodes.

Screening results in health care workers and environment

During the endemic phase, no MRSA was isolated from 60 HCW screenings. From 118 environmental samples taken, MRSA was isolated twice from a stethoscope. During the epidemic phase, no MRSA was isolated from 42 environmental samples; 142 HCWs were screened, nine persons (6.3%, five nurses, three nursing students and one ward assistant responsible for environmental cleaning and disinfection procedures in the NICU) were identified as nasal and/or throat MRSA carriers. However, only seven isolates (4.9%) were related to the outbreak isolate. All staff members were successfully decolonized. Two nurses had to undergo a second cycle of mupirocin, and one nurse suffering from atopic dermatitis was successfully decolonized after three cycles. Due to underlying bronchial asthma, disinfection-related coughing and throat colonization with MRSA, the ward assistant was assigned to a workplace outside of the neonatology department.

Phage typing, PFGE and in vitro antibiotic sensitivity

All of the 27 neonatal and seven of the nine HCW-related outbreak isolates showed strong reactions in phage group III (47, 54, 75, 77, 83) and group M (81) of the IPS, with the additional regional phages (D11, 16, 192) and the

MRSA phage set (MR8, MR12 and M3). This pattern characterized the isolates as epidemic Rhine-Hessen clone of hospital-acquired MRSA. All isolates tested showed a pattern characteristic to the epidemic Rhine-Hessen clone MRSA.

All strains were resistant to ß-lactams, erythromycin, clindamycin and ciprofloxacin in vitro, in accordance with the usual phenotype of hospital-acquired MRSA.

Decolonization success in neonates

Of the 27 neonates with positive MRSA cultures, eight patients were discharged or transferred to other units as 'MRSA positive' before completion of decolonization. Of the 19 remaining patients with available results after decolonization, nine (47%) showed persistent colonization and ten (53%) were decolonized successfully.

One standard cycle of mupirocin and polyhexanide bathing was sufficient to decolonize seven children, one of whom received additional oral vancomycin. Two patients were decolonized after two cycles. More than two cycles were applied to three children, but without success.

Gastrointestinal colonization was detected in 15 of 27 (56%) neonates. Sufficient follow-up data after decolonisation was available for ten patients, three of whom were decolonised successfully (i.e., 30% vs. 78% in absence of gastrointestinal colonization; RR=0.39; 95% confidence interval, 0.14–1.06).

Vancomycin was applied orally to seven patients with GI-positive cultures. In only one of these, a successful decolonization was confirmed by cultures.

Discussion

The hospital-acquired MRSA outbreak in our NICU was caused by a single clone, confirmed by phage and PFGE-typing. We assume a combination of indirect patient-to-patient transmission and spread through the colonization of health care workers as previously described [8], which may have been supported by insufficient adherence to standard hygiene procedures and the ability of MRSA to persist for long periods on contaminated surfaces [9, 10]. Fortunately, no patient other than the index case suffered a serious infection due to MRSA. During an initial endemic phase (February to October) with a mean of 1.7 (0–4) new MRSA-cases/month, standard preventive measures were taken. The installation of an outbreak management team (OMT), prompted by a sharp increase in incidence, including the decision-makers of the unit, helped to implement measures successfully. We assume that our comprehensive approach targeting patients, staff and environment as possible reservoirs for MRSA (cohorting on isolation wards, comprehensive disinfection measures, extensive screening and decontamination strategy) was important for the successful termination of the outbreak.

Since December 2005, one month after installation of the OMT, no single new MRSA-case was registered for more than a year.

In contrast to other authors who declared nasal swabs as the 'one and only' screening site [5], our data show a higher sensitivity of stool cultures, in particular in the detection of persisting carriage, and suggest screening of nares, throat and stool. The gastrointestinal tract has been identified as an important reservoir of MRSA in persistent carriers in adult patients [11–15] and in a pediatric intensive care unit [16]. Contrary to the data reviewed by Acton et al., rectal swabs were clearly inferior to stool samples in detecting persistent colonization [15].

We assume that gastrointestinal colonization in neonates contributes significantly to the overall risk of environmental contamination, particularly if standard hygiene procedures and routine surface disinfection are not strictly followed [9, 10]. The approach to administer vancomycin to GI-positive patients [16] has been disappointing in our patients.

In screening for persistent colonisation, we advise against using less than three swabs per site, since in the majority of episodes only one swab out of three yielded MRSA. Although health care workers (HCW) have been involved in outbreaks [8], the role of routine HCW-screening and the question how to handle a positive result in a HCW is still a matter of debate. However, screening and decolonization of HCWs and exclusion from direct patient care may play an important role. The detection of MRSA colonization in a woman (presenting with bronchial asthma, throat MRSA colonization, and disinfection-associated coughing) responsible for surface cleaning and disinfection efforts in our unit underlines the necessity to include more than just nurses and physicians into the screening population.

In summary, we conclude that a comprehensive outbreak management including the decision-makers can successfully terminate an outbreak in a NICU setting within a short time. Thorough screening of nares, throat and stool for MRSA, repeated three times, is necessary for correct cohorting of patients. However, gastrointestinal decolonization in neonates seems difficult.

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Early phase evaluation of SQ109 alone and in combination with rifampicin in pulmonary TB patients.

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Early phase evaluation of SQ109 alone and in combination with rifampicin in pulmonary TB patients

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Objectives: SQ109, an asymmetrical diamine, is a novel anti-TB drug candidate. This first study in patients was done to determine safety, tolerability, pharmacokinetics and bacteriological effect of different doses of SQ109 alone and in combination with rifampicin when administered over 14 days.

Patients and methods: Smear-positive pulmonary TB patients were randomized into six groups of 15 to receive once-daily oral treatment with 75, 150 or 300 mg of SQ109, rifampicin (10 mg/kg body weight), rifampicin plus 150 mg of SQ109, or rifampicin plus 300 mg of SQ109 for 14 days. Patients were hospitalized for supervised treatment, regular clinical, biochemical and electrocardiographic safety assessments, pharmacokinetic profiling and daily overnight sputum collection.

Results: SQ109 was safe and generally well tolerated. Mild to moderate dose-dependent gastrointestinal complaints were the most frequent adverse events. No relevant QT prolongation was noted. Maximum SQ109 plasma concentrations were lower than MICs. Exposure to SQ109 (AUC_{0-24}) increased by drug accumulation upon repeated administration in the SQ109 monotherapy groups. Co-administration of SQ109 150 mg with rifampicin resulted in decreasing SQ109 exposures from day 1 to day 14. A higher (300 mg) dose of SQ109 largely outweighed the evolving inductive effect of rifampicin. The daily fall in log cfu/mL of sputum (95% CI) was 0.093 (0.126–0.059) with rifampicin, 0.133 (0.166–0.100) with rifampicin plus 150 mg of SQ109 and 0.089 (0.121–0.057) with rifampicin plus 300 mg of SQ109. Treatments with SQ109 alone showed no significant activity.

Conclusions: SQ109 alone or with rifampicin was safe over 14 days. Upon co-administration with rifampicin, 300 mg of SQ109 yielded a higher exposure than the 150 mg dose. SQ109 did not appear to be active alone or to enhance the activity of rifampicin during the 14 days of treatment.

Keywords: safety, pharmacokinetics, bactericidal activity, Phase 2, tuberculosis, treatment, early bactericidal activity

Introduction

A more effective treatment regimen for drug-susceptible TB is needed to control the TB epidemic. SQ109, a new anti-TB drug candidate, was identified from a combinatorial chemistry programme around the diamine pharmacophore of ethambutol.[1] Known SQ109 mechanisms of action include inhibition of MmpL3, a monomycolate transporter involved in *Mycobacterium tuberculosis* (MTB) cell wall assembly,[2] effects on energy production and inhibition of efflux systems in MTB.[3] These mechanisms are all

distinct from that of ethambutol[4] and cross-resistance with SQ109 has not been observed.[5] *In vitro*, SQ109 shows synergy with isoniazid, bedaquiline and sutezolid,[6–8] and a marked synergy with rifampicin.[5] In a mouse model of chronic TB, SQ109 at 10 mg/kg was more effective than ethambutol at 100 mg/kg[9] and increased the bactericidal activity of standard four-drug treatment, with superior activity first becoming evident after 4 weeks of therapy.

SQ109 is known to be metabolized by cytochrome P450 isoenzymes CYP2D6 and CYP2C19,[10] and to distribute rapidly and extensively to murine tissues including the liver, lung, spleen, and kidney, resulting in tissue concentrations that for lung tissue were at least 40-fold above plasma levels.[10,11] In humans, co-administration with rifampicin could potentially reduce SQ109 exposure by induction of cytochrome P450 isoenzymes, mainly CYP2C19.[10,12,13]

In Phase 1, healthy volunteers had received single and multiple doses of SQ109 up to 300 mg daily for up to 14 days; these were safe and well tolerated with some events of mild to moderate gastrointestinal discomfort, but no apparent QT prolongation or other cardiovascular, hepatic, renal or respiratory safety signals.[5] SQ109 showed dose-dependent increases in total exposure and reached steady state by day 11. Drug plasma levels in Phase 1 participants after 14 days of 300 mg daily showed a geometric mean C_{max} of 23.0 ng/mL (17.5–37.9 ng/mL) and a geometric mean AUC_{0-24} of 140 ng·h/mL (94.8–274 ng·h/mL). The geometric mean elimination half-life ($t_{1/2}$) was 48.7 h in this study, and had been found to increase with dose and time of administration in previous studies. The apparent volume of distribution during the elimination phase (Vz/F,ss) was very large and estimated to be 123 000 L (Sequella, Inc., unpublished data). Plasma protein binding in humans is 14%–24%.[10,12,13]

Based on the favourable safety profile, the doses used in Phase 1 studies were chosen for Phase 2 work. Human exposure at a daily dose of 300 mg measured as AUC_{0-24} at 14 days was similar to AUC_{0-24} in mice administered a single dose of 25 mg/kg.[13] The minimum effective dose in the mouse model of TB is 10 mg/kg/day administered for 28 days.[13] The C_{max} in healthy humans was generally lower than the C_{max} in mice at all doses.[13] Matching AUC was seen as the more relevant parameter, and, based on the favourable safety profile, the 300 mg dose was chosen for Phase 2 work, along with lower doses, to show dose–response effects.[5,11]

In development programmes for anti-TB drugs, the first study in patients usually is short, often with a duration of 2 weeks, in relatively healthy smear-positive pulmonary TB patients who receive either monotherapy with the experimental agent or combinations of agents with mostly uncertain anti-TB activity, which is deemed ethically acceptable for this limited period of time. The overall aim of such studies is to assess the dose of the drug to be used in follow-up studies. In order to achieve this, these studies explore safety and pharmacokinetics as well as changes in sputum bacterial counts during treatment, the 14 day early bactericidal activity (EBA). Such studies have been used in most recent evaluations of novel drugs,[14–17] and are included in the US FDA's draft guidance on drug development for pulmonary TB.[18]

This study assessed the safety, tolerability, pharmacokinetics and 14 day EBA of SQ109 alone and in combination with rifampicin in patients with pulmonary TB to explore the potential synergistic activity and pharmacokinetic interactions of these compounds in the first 14 treatment days.

Patients and methods

Study design and endpoints

Study LMU-IMPH-SQ109-01 was a partially blinded, randomized, controlled trial of the safety, tolerability, pharmacokinetics and bactericidal activity of SQ109 with or without co-administration of rifampicin. The study was registered in the Pan-African Clinical Trials Registry (pactr.org; PACTR201009000252144) and clinicaltrials.gov (NCT01218217) prior to commencement. The primary safety endpoint was the proportion of subjects with serious adverse events and the number of subjects who discontinued due to adverse event(s). The primary pharmacokinetic endpoint was the total exposure to SQ109 in plasma over the 24 h dosing interval (AUC_{0-24}) as assessed after 14 days of administration of SQ109 with or without rifampicin. The primary and secondary efficacy endpoints were the change in $\log_{10}$ cfu counts on solid culture media and the change in time to culture positivity (TTP) in liquid culture over time, respectively.

Patients and ethics

The study was conducted in three clinical sites in Cape Town (Task Applied Science, Intercare Hospital, Bellville, Brooklyn Chest Hospital, Ysterplaat, and University of Cape Town Lung Institute, Centre for Tuberculosis Research Innovation, Mowbray). The South African Medicines Control Council and the relevant ethical boards approved the study. All study participants provided written informed consent for study participation.

Patients were considered eligible for participation if they had newly diagnosed, uncomplicated, untreated pulmonary TB evidenced with sputum at least 1+ on an auramine-stained smear and a GeneXpert MTB/RIF test (Cepheid, Sunnyvale, CA, USA), indicating the presence of MTB complex and rifampicin susceptibility. Patients were excluded if they were HIV-positive and on antiretroviral treatment or had <250 CD4 cells/mm^3. Further excluded were patients with body weight <40 or >90 kg and conditions that increased the risk of important medical events, such as known or suspected drug addiction, insulin-dependent diabetes, other significant comorbidities that would have increased the risk of study participation, or relevant ECG alterations as summarized by the E14 guidance of the International Conference on Harmonization.[19]

Study treatments and procedures

A total of 90 subjects were assigned, using blocked randomization stratified by site, to one of six equally sized treatment groups: 75 mg of SQ109; 150 mg of SQ109; 300 mg of SQ109; 150 mg of SQ109 plus 10 mg/kg rifampicin; 300 mg of SQ109 plus 10 mg/kg rifampicin; and 10 mg/kg rifampicin. Study treatment was administered once daily in the morning on an empty stomach, after a minimum fast of 8 h, for 14 days. SQ109 tablets of 150 mg and matching placebo were provided by Sequella, Inc. (Rockville, MD, USA). Rifampicin was obtained from Svizera (Almere, the Netherlands) in capsules of 150 mg and dosed according to body weight with 450 mg doses for participants weighing 40–54 kg, 600 mg for those weighing 55–70 kg and 750 mg for those weighing $\geq$71 kg. Participants, staff and sponsor were blinded to SQ109 allocation and dose by the use of matching placebo tablets. There was no attempt to blind staff or participants to allocation to rifampicin.

Participants were hospitalized for at least 2 days before treatment initiation and discharged on the day after the last dose of study medication following initiation of a full course of standard anti-TB treatment. Follow-up visits were conducted 2 and 13 days after discharge. The safety assessment battery included daily physical examinations and recording of vital signs. Upon regulatory advice, a neurological examination at baseline and after completion of experimental treatment was included. As the drug shares a common core structure with ethambutol, vision testing with Snellen and Ishihara charts was repeated after both 7 and 14 days of treatment to record eventual toxicity. Samples for haematology,

coagulation profile, serum chemistries and urinalysis were collected at screening, on study days 2, 6, 10 and 15 and on the last follow-up visit. Adverse events were graded according to the US National Cancer Institute's Common Terminology Criteria for Adverse Events (CTCAE), Version 4.02, published 15 September 2009. Twelve-lead ECGs were obtained in triplicate at screening, pre-dose, 1, 2 and 4 h after dosing on study day 1 and 2 h after dosing on study days 7 and 14. According to ICH E14 guidelines, QT intervals were corrected following Bazett's (QTcB) and Fridericia's (QTcF) formulae for each ECG.[19]

On treatment days 1, 7 and 14, serial venous blood samples were collected just prior to and 0.5, 1, 2, 3, 4, 6, 8, 12 and 24 h after witnessed intake of SQ109 and/or rifampicin on an empty stomach. Plasma was immediately separated, stored at −80°C and transported on dry ice for bio-analysis. Total (protein-bound plus unbound) plasma concentrations of SQ109 were measured using a validated LC/MS/MS method by Cetero Research (Houston, TX, USA). The limit of quantification for SQ109 in plasma was 0.5 ng/mL. Rifampicin plasma concentrations were measured at Radboud University Medical Centre (Nijmegen, the Netherlands) with a validated assay, modified after a previously described assay.[20] The pharmacokinetic analysis population included all dosed subjects with evaluable pharmacokinetic data, but excluded subjects if their derived pharmacokinetic parameters were considered invalid due to relevant missing values.

Microbiological testing was done centrally in the Department of Medical Biochemistry, Faculty of Medicine and Health Sciences, Stellenbosch University, Cape Town, South Africa, for all clinical sites. Sputum for cfu counts of MTB on solid media and determination of time to a positive signal (TTP) in liquid culture medium was collected daily for 16 h overnight from 2 days before treatment initiation to the last treatment day; collections were completed prior to administration of the day's therapy and transported to the laboratory at 2–8°C for processing. Briefly, sputum was homogenized by magnetic stirring. Dithiothreitol (1:20 dilution; Sputasol, Oxoid, Cambridge, UK) was added to a maximum of 10 mL of homogenized sputum in equal volume. Sputum was vortexed for 20 s and left to digest at room temperature for 20 min. For cfu counting, 1 mL of this digested sputum was used to prepare a range of 10-fold dilutions from 10^0 to 10^{-5}. From each dilution, 100 μL was plated in quadruplicate on 7H11 agar plates (BD, Franklin Lakes, NJ, USA) that contained 200 U/mL polymyxin B, 10 μg/mL amphotericin B, 100 μg/mL ticarcillin and 10 μg/mL trimethoprim (Selectatab; Mast, Merseyside, UK). Numbers of cfu were counted after 3–4 weeks of incubation at 37°C at the dilution yielding 20–200 visible colonies. For the TTP measurement, we used a standardized liquid culture system (Bactec Mycobacteria Growth Indicator Tube; MGIT 960, BD). Briefly, homogenized sputum was decontaminated (MycoPrep; BD), centrifuged and resuspended, and 0.5 mL of the resulting 2 mL was used for incubation in duplicate.

Cultures from baseline and the last available overnight sputum collections were used for testing susceptibility to isoniazid, rifampicin, ethambutol and streptomycin (MGIT; Becton Dickinson). The MIC of SQ109 was determined for the same cultures using the broth microdilution method at a concentration range of 0.03–32 μg/mL. MTB complex speciation was done by PCR (Accuprobe, Gen-Probe, San Diego, CA, USA).[21]

Statistical analysis

This was a descriptive study with no inferential statistics or hypothesis testing. Patients were included in the ITT population if they had received at least one dose of study drug and in the PP population if they had met all inclusion criteria and no exclusion criterion and had taken all study medication according to protocol. Safety analysis was done on the ITT population. Pharmacokinetic parameters were calculated using non-compartmental analysis and actual blood sampling times with SAS® for Windows® Version 9.3 and Winnonlin version 5.3 (Pharsight Corp., St Louis, MO, USA). Linear mixed-effects repeated-measures models were used to model the decline in $\log_{10}$ cfu and TTP over time, including the pre-treatment values as responses. Visit day was first included as a discrete variable to estimate mean $\log_{10}$ cfu at each visit without assuming a particular form for the decline over time to describe the data. Visit day was then included in the model as a continuous variable to estimate the decline in $\log_{10}$ cfu over time (EBA). Negative culture results were excluded from the models. To estimate the effect of excluding negatives, sensitivity analyses were conducted including negative cultures imputed with 0.5 $\log_{10}$ cfu or 42 days for each model. Baseline characteristics were compared across groups using Fisher's exact test for binary variables and the *F*-test and analysis of variance for continuous variables. Linear regression was used to explore microbiological and pharmacokinetic predictors of individual-level EBA. $P<0.05$ was considered evidence for an association.

Results

Patients

Between December 2010 and August 2011, a total of 136 patients were screened, of whom 90 were randomized and received at least one dose of study medication. The disposition of all of the patients is described in Figure 1. Baseline characteristics were comparable between groups, with non-significant differences in gender distribution and baseline bacterial load. Baseline characteristics are summarized in Table 1.

Two patients did not complete the period of drug intake. One was withdrawn due to rifampicin resistance discovered at screening that had been overlooked (150 mg of SQ109), and one patient for exacerbation of pre-existing epigastric pain that made compliance with drug intake difficult. Endoscopy after withdrawal identified oesophageal candidiasis as the likely culprit. The adverse event was judged related to study drug by the investigator (150 mg of SQ109; Figure 1). A third patient was excluded from ITT and PP efficacy analyses as all sputum samples were contaminated (150 mg SQ109 plus rifampicin). In two further patients, rifampicin dosing errors were made, so in total 85 patients were included in the PP analysis.

Safety

The only serious adverse event was a case of fatal haemoptysis that occurred 11 days after the last dose of 300 mg of SQ109 plus rifampicin. The investigator considered this as unrelated to study treatment. There was one grade 3 event of drug-induced hepatitis, which developed 12 days after discontinuing SQ109 treatment and starting standard anti-TB treatment. This was also assessed as unrelated to study treatment (Table 2). The most frequently reported adverse events were nausea or gastrointestinal distress (56%). This was more frequent in the groups receiving SQ109-containing treatments and with increasing doses of SQ109 when given alone (Table 2). There were no adverse events related to ECG, and no QTcB or QTcF prolongations beyond 500 ms, or increases of >60 ms compared with baseline. Mean QTcF increase from baseline was 5.1 ms after 7 days of treatment (95% CI 1.7–8.6) and 3.4 ms after 14 days of treatment (95% CI −0.9 to 7.6). In comparison, in the rifampicin control group the mean QTcF increase was 8.8 ms (95% CI −2.4 to 15.3) at day 7 and 11.0 ms (95% CI 4.7–17.3) at day 14. There were no significant changes in vibration sense, visual acuity measurements or other neurological examination parameters.

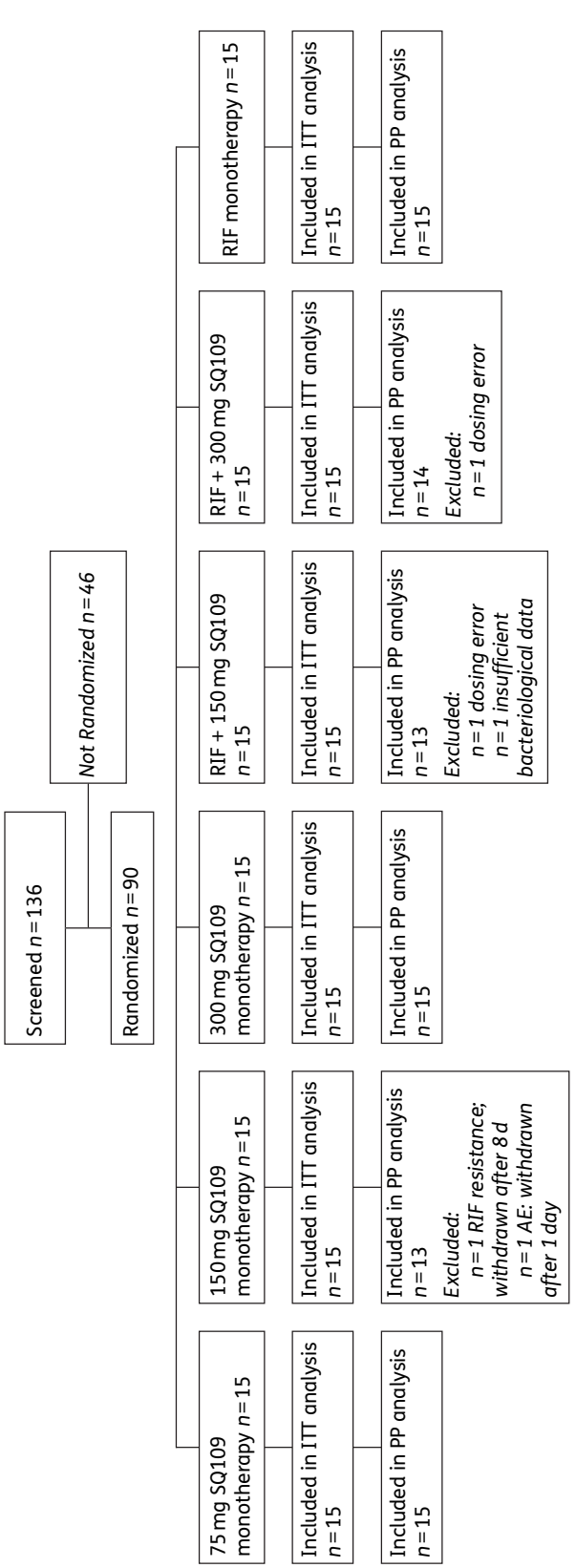

Figure 1. Recruitment and analysis flow chart. Primary reason for non-randomization: positive urine drug screen (14), CD4 cell count $<250/mm^3$ (7), abnormal laboratory values (6), rifampicin resistance (6), insufficient bacterial load by smear microscopy (5), ECG (5), incomplete medical history (1), withdrew informed consent (1) and uncontrolled diabetes mellitus (1). AE, adverse event (see the text); RIF, rifampicin.

Pharmacokinetics

In each of the SQ109 monotherapy groups, repeated administration of SQ109 resulted in an increase in the geometric mean AUC_{0-24} of SQ109 from day 1 to day 7 to day 14 (Table 3). Comparison of the three SQ109 monotherapy groups showed that increasing doses of SQ109 (at day 1, day 7 or day 14) led to increases in geometric mean SQ109 AUC_{0-24} values. At the 150 mg dose, co-administration of SQ109 with rifampicin led to a progressive reduction in SQ109 AUC_{0-24} and C_{max} over time compared with SQ109 administered alone. Upon co-administration of rifampicin with the highest dose (300 mg) of SQ109 the geometric mean AUC_{0-24} to SQ109 increased from day 1 to day 7, but then also decreased from day 7 to 14. In effect, a decrease in AUC $_{0-24}$ from day 7 to day 14 occurred in 12/15 participants and the geometric mean SQ109 AUC_{0-24} day 14/day 7 ratio was 0.84. There was wide inter-individual variability in AUC_{0-24} and C_{max} of SQ109, especially at the 300 mg dose [Table 3 and Figure S1 (available as Supplementary data at *JAC* Online)]. No effect of SQ109 on exposure to rifampicin was seen.

Bactericidal activity

All strains were identified as MTB, and all patients but one were infected with MTB isolates that were susceptible to rifampicin. The MIC of SQ109 was as expected from preclinical testing (median 125 mg/L; range <30–500 mg/L) and did not differ by more than one concentration step before and after treatment. The cfu counts on solid agar yielded 1257 positive results; in 163 samples no data were available due to culture contamination, absence of growth or missing results. There was a linear decline in $\log_{10}$ cfu in every treatment group receiving rifampicin. In contrast, there was no apparent decline in any of the SQ109 monotherapy groups (Figure 2a and c). The sensitivity analysis imputing negative cultures with 0.5 $\log_{10}$ cfu or 42 days did not differ from the results excluding negative cultures (data not shown). CIs for bactericidal activity overlapped for all SQ109 monotherapy groups, as well as for all rifampicin-receiving groups. The histogram of TTP was found to be positively skewed and did not closely follow the normal distribution. Log_{10}(TTP) was more symmetrical and fitted the normal distribution. Therefore, the mean TTP was taken on the log scale (geometric mean) and $\log_{10}$(TTP) was included as the dependent variable in the models below. The mean was taken from two duplicates. There was a linear increase from baseline $\log_{10}$(TTP) in the treatment groups containing rifampicin but not in any of the SQ109 monotherapy groups (Figure 2b and d). Baseline bacterial load measured in both cfu and TTP was not associated with the change in bacterial load over time for individual patients (data not shown).

Discussion

Our study has shown that incremental daily doses of up to 300 mg of SQ109 were safe when administered with or without rifampicin for 14 days. SQ109 plasma levels remained below the MICs for the infecting organisms. There was no apparent antimycobacterial effect of SQ109 over the first 14 days of treatment at drug exposures achieved with doses up to 300 mg.

Table 1. Baseline demographic and clinical characteristics

	75 mg SQ109	150 mg SQ109	300 mg SQ109	RIF and 150 mg SQ109	RIF and 300 mg SQ109	RIF	All
Total patients randomized, *n*	15	15	15	15	15	15	90
Male, *n* (%)	8 (53)	12 (80)	9 (60)	6 (40)	8 (53)	10 (67)	53 (59)
HIV positive, *n* (%)	2 (13)	1 (7)	3 (20)	1 (7)	1 (7)	1 (7)	9 (10)
Weight (kg), median (IQR)	51.8 (44.6–56.3)	50.2 (43.6–54.0)	51.7 (50.4–62.9)	50.1 (46–58.3)	52.4 (46.4–60.3)	50.4 (48.0–54.1)	50.9 (46.4–56.2)
Age (years), median (IQR)	30.7 (23.4–44.6)	31 (23.7–43.3)	26.2 (21.1–46.2)	24.2 (21.1–37.7)	27.7 (22.4–40.2)	37.8 (27.3–49.2)	30.4 (23.4–40.7)
Log_{10} cfu, mean (95% CI)	5.5 (4.9–6.2)	5.3 (4.8–5.8)	5.6 (5.1–6.1)	6.3 (5.9–6.7)	5.9 (5.5–6.3)	5.5 (4.9–6.0)	5.7 (5.5–5.9)
TTP (days), median (IQR)	4.7 (3.4–5.4)	4.4 (3.9–5.2)	4.9 (4.1–6.3)	4.3 (3.5–5.6)	4.1 (3.3–4.7)	4.3 (3.5–5.6)	4.5 (3.8–5.2)

RIF, rifampicin.
There were no statistically significant differences ($P<0.05$) in any of these characteristics between treatment groups.

Table 2. Summary of all adverse events registered up to day 28, reported by organ system and treatment arm

Number of adverse events in category	75 mg SQ109, *n*=15	150 mg SQ109, *n*=15	300 mg SQ109, *n*=15	RIF and 150 mg SQ109, *n*=15	RIF and 300 mg SQ109, *n*=15	RIF only, *n*=15	Total, *n*=90
Gastrointestinal disorders							
mild	4	2	10	4	4	2	
moderate	1	5	8	2	2	2	
total gastrointestinal disorders	5	7	18	6	6	4	46
Nervous system disorders	1	1	2	2	1	2	9
Respiratory, thoracic and mediastinal disorders	1	1	0	2	3[a]	1	8
Skin and subcutaneous tissue disorders	0	2	0	0	1	1	4
Musculoskeletal and connective tissue disorders	0	2	2	0	0	0	4
Common cold	0	1	0	0	0	0	1
Temporary reduction in visual acuity	1	1	0	0	0	0	2
Raised liver enzymes	1[b]	2	1	0	0	2	6
Raised lipase	0	0	0	0	0	1	1
Total	9	17	23	10	11	11	81

All adverse events were mild to moderate in severity, except those mentioned in the footnotes. Classification and grading was done following Common Toxicity Criteria for Adverse Events (CTCAE), version 4.0.[31]
[a]Includes one death due to massive haemoptysis (unrelated).
[b]Includes one grade 3 adverse event, unrelated to study treatment (see the text).

Mild to moderate, dose-dependent gastrointestinal discomfort was the most frequently seen adverse event. Average QTcF changes with SQ109 were less than those seen with rifampicin, and remained below the threshold of regulatory concern.

The results of the pharmacokinetic analysis in the SQ109 monotherapy groups showed that the AUC_{0-24} of SQ109 increased from day 1 to day 7 to day 14, which can be ascribed to accumulation of SQ109 with repeated dosing. Increases in SQ109 doses resulted in increases in SQ109 exposure. Upon co-administration of rifampicin with the *lower* SQ109 dose of 150 mg, the AUC_{0-24} of SQ109 decreased from day 1 to day 7 to day 14. Induction of metabolic or transporter enzymes by rifampicin apparently outweighs the accumulation of SQ109 when administered in a lower dose and this inductive effect increases over time. Indeed, maximum induction by rifampicin is expected after 7–14 days.[12] Upon co-administration of rifampicin with the *higher* dose of SQ109 (300 mg), the geometric mean AUC_{0-24} to SQ109 increased from day 1 to day 7, but then decreased from day 7 to 14. Thus it appears that accumulation of SQ109 administered at a higher dose is able to outweigh

Table 3. Pharmacokinetics of SQ109, rifampicin and SQ109 combined with rifampicin

	SQ109 alone			SQ109 and RIF				RIF alone
	75 mg	150 mg	300 mg	SQ109 150 mg	RIF 10 mg/kg	SQ109 300 mg	RIF 10 mg/kg	RIF 10 mg/kg
Number of patients	15	12	14	15	15	15	15	14
Day 1								
AUC_{0-24}[a]	58.9[b] (10.2–152.6)	103.4[c] (46.0–586.2)	183.7 (32.0–599.5)	146.3[d] (49.9–294.3)		181.2 (54.7–436.5)		
C_{max}[a]	15.5 (2.2–100.9)	20.9 (5.3–66.1)	43.3 (5.1–119.5)	28.5 (4.8–103.3)		43.7 (13.4–159.2)		
T_{max} (h)	0.9 (0.0–1.9)	1.0 (0.9–2.92)	1.0 (0.5–1.9)	0.9 (0.5–3.0)		1.0 (0.4–4.0)		
CL/F (L/h)	1274[b] (491–7324)	1451[c] (256–3261)	1633 (500–9390)	1025[d] (510–3004)		1656 (687–5487)		
Day 7								
AUC_{0-24}[a]	71.5[e] (21.2–176.3)	136.3 (31.2–2025.2)	240.8 (58.4–666.5)	101.2 (33.5–219.7)		252.2 (38.1–672.4)		
C_{max}[a]	16.8 (4.0–68.5)	24.9 (6.6–205.0)	39.1 (9.2–122.1)	19.2 (4.5–53.7)		44.7 (8.0–150.1)		
T_{max} (h)	0.5 (0.0–2.9)	0.9 (0.5–2.0)	0.9 (0.50–1.9)	1.0 (0.4–6.0)		1.0 (0.9–4.0)		
CL/F (L/h)	1049[e] (425–3533)	1101 (74–4814)	1246 (450–5141)	1483 (683–4483)		1189 (446–7880)		
Day 14								
AUC_{0-24}[a]	85.1[e] (21.5–165.0)	152.5 (41.0–1369.9)	268.5 (111.6–989.4)	64.4 (24.8–141.2)	35.0 (16.2–64.7)	212.5 (31.6–809.9)	30.1 (15.6–55.0)	32.7 (20.3–58.6)
C_{max}[a]	14.6 (4.0–45.3)	27.4 (9.4–139.9)	45.9 (15.7–146.0)	10.6 (4.2–32.8)	8.2 (3.8–13.4)	35.8 (8.3–679.1)	7.2 (3.2–12.1)	8.0 (5.1–16.2)
T_{max} (h)	0.9 (0.0–2.0)	1.0 (0.4–2.0)	1.0 (0.5–5.9)	1.0 (0.4–3.0)	2.1 (1.9–4.0)	1.0 (0.4–3.0)	3.0 (1.9–5.9)	1.5 (1.0–3.0)
CL/F (L/h)	881[e] (455–3485)	984 (109–3658)	1117 (303–2687)	2328 (1062–6051)	14.6 (9.5–46.3)	1411 (370–9497)	16.4 (8.2–38.4)	14.6 (8.4–22.2)

RIF, rifampicin; CL/F, apparent clearance.
Parameters are presented as geometric means and ranges, apart from T_{max}, which is presented as median and range.
Elimination rate constant and elimination half-life could be assessed correctly in only a minority of patients and are not displayed.
[a]Units for AUC_{0-24} are h·ng/mL for SQ109 and h·mg/L for rifampicin, and units for C_{max} are ng/mL for SQ109 and mg/L for rifampicin.
[b]$n=10$.
[c]$n=11$.
[d]$n=12$.
[e]$n=13$.

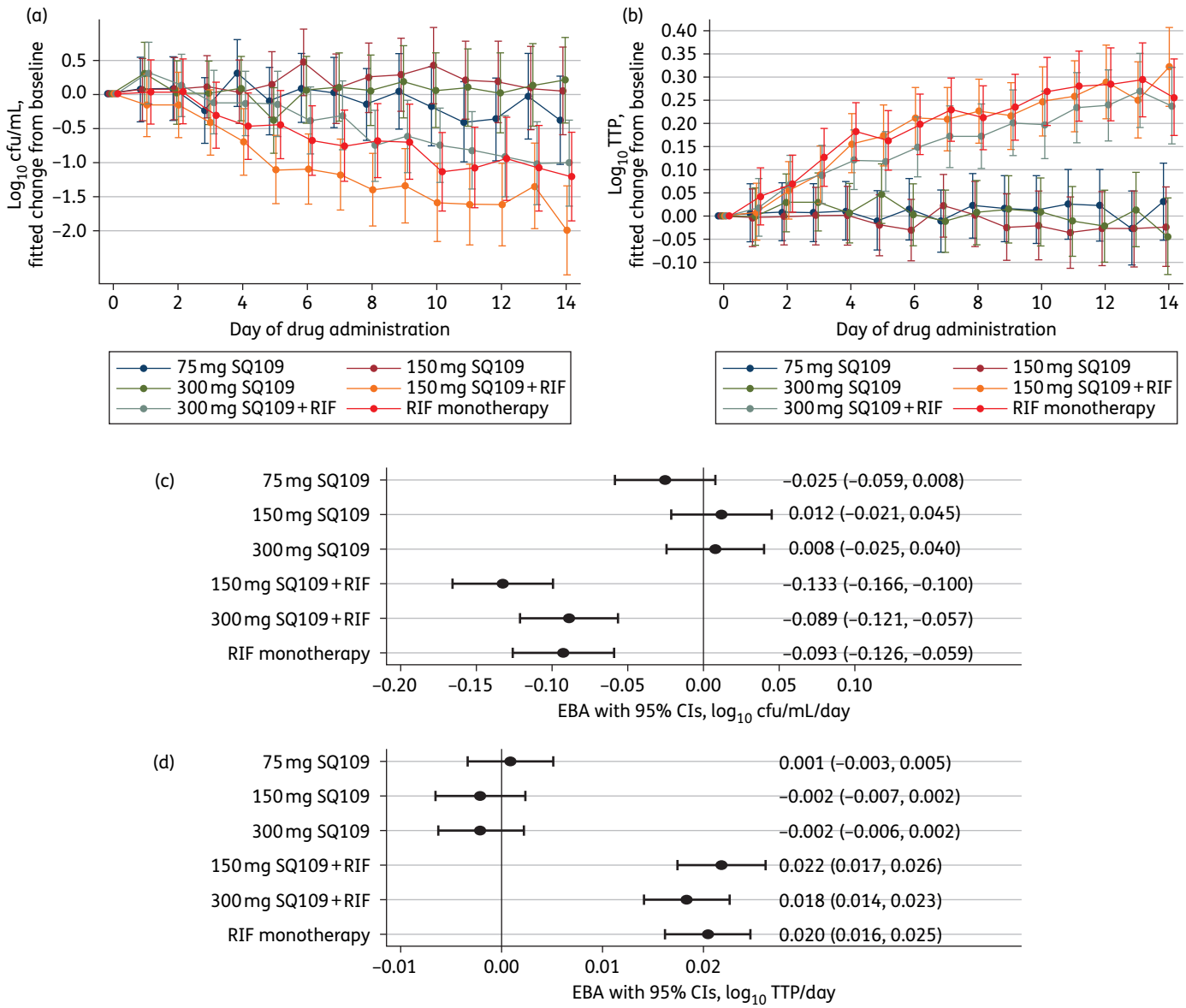

Figure 2. Fitted estimates of differences from mean baseline $\log_{10}$ cfu on solid agar (a) and $\log_{10}$ TTP in liquid culture (b) over time. Linear mixed-effects repeated-measures models were used to model the decline in $\log_{10}$ cfu and $\log_{10}$ TTP over time including the pre-treatment values as responses. (a) Mean $\log_{10}$ cfu is estimated for each visit day, without assuming a particular form for the decline over time to describe the data. Bars show 95% CIs. (b) Log_{10} TTP is estimated for each visit day without assuming a particular form for the decline over time to describe the data. Bars show 95% CIs. (c) Estimated EBA and CIs from daily sputum colony counts on solid media from a mixed-effects model assuming a linear decline. Bars show 95% CIs; average values of change per day (limits of 95% CIs in brackets) are displayed next to the plot. (d) Estimated EBA and CIs from daily TTP from a mixed-effects model assuming a linear decline. TTP was $\log_{10}$ transformed for this analysis. Bars show 95% CIs; numerical values of average change per day (limits of 95% CIs in brackets) are displayed next to the plot. An EBA of 0.02 $\log_{10}$ TTP/day, for example, corresponds to an increase in TTP from 10 days to 10.47 days over 1 day of patient follow-up, or an increase in TTP from 5 days to 5.24 days over 1 day of patient follow-up. RIF, rifampicin.

the evolving metabolic induction of rifampicin, yet the higher SQ109 dose does not compensate for the full induction that is achieved beyond 1 week of treatment with rifampicin. Pharmacokinetic studies have shown that CYP2D6, one of the enzymes responsible for metabolizing SQ109,[10] is modestly (if at all) induced by rifampicin.[12] Thus, the rifampicin effect is probably due to induction of CYP2C19, the other enzyme involved in the metabolism of SQ109.[10,13] Some caution is warranted when considering the effect of rifampicin on SQ109 when comparing study groups. The number of participants in each group was small, and wide inter-patient variability in exposure to SQ109 was observed. For example, patients in the monotherapy group receiving 150 mg of SQ109 started off with a lower average AUC_{0-24} to SQ109 than those in the group receiving 150 mg of SQ109 with rifampicin co-administration. This variability can be explained at least partly by genetic polymorphisms that occur both in CYP2D6 and CYP2C19.[10,22] In addition, extensive, intermediate and poor metabolizers for these cytochromes may also

be at different risks of inhibition or induction by co-administered drugs.[23,24] The average pharmacokinetic data presented in this study may therefore conceal possible differences between genetically different patient subgroups. Unfortunately, genotypes for CYP2D6 and CYP2C19 were not determined in the study population. It was concluded that SQ109 should be administered at a minimum dose of 300 mg once daily in follow-up studies, as this dose resulted in the highest exposures even with rifampicin co-administration. There appeared to be no large or dose-related effect of SQ109 on exposure to rifampicin, which agrees with data from murine studies.[5]

No bactericidal effect of SQ109 alone or additional to rifampicin was detected during the 14 days of administration. The EBA for rifampicin alone was −0.093 $\log_{10}$ cfu/mL·day (95% CI −0.126 to −0.059), which is consistent with the only reported rifampicin 10 mg/kg monotherapy study over 14 days (EBA day 0 to day 14, −0.113 $\log_{10}$ cfu/mL·day).[25] The variation observed with TTP was less than with cfu, so that significant group effects were discovered with TTP more readily than with cfu.[26] The significance of the lack of early bactericidal effect for SQ109 alone in the first 2 weeks of treatment is uncertain and allows several possible explanations.

Firstly, concentrations in human lung lesions may have been too low. Maximum plasma concentrations remained well below the MICs, so an accumulation in lungs similar to that observed in mice would have been necessary. Murine lung and spleen tissue concentrations were at least 40-fold higher than in plasma, far above the MIC for MTB after even a single oral dose of 25 mg/kg,[5,11] and reached steady state after 14 days at the 10 mg/kg dose.[11] The tissue-to-plasma partition coefficient in humans is unknown, and the pharmacokinetic parameter best associated with this accumulation in mice is not well defined. Plasma AUCs in patients reached levels similar to those in mice, but it is still possible that a presumed process of accumulation depends more on C_{max}, which was lower in humans than in mice.

Secondly, the drug may not reach the compartment where the bacteria that are measured in sputum reside. It was found that SQ109 accumulates within cells and reportedly acts on intracellular bacteria at concentrations of $\geq$1 μg/mL in the whole blood assay.[11,27] The observed efficacy in mouse models could be attributed mostly to intracellular action, since SQ109 has only been characterized in models of TB where the bacilli are contained intracellularly[28] as opposed to the C3HeB/FeJ mouse model of TB, which forms necrotic granulomas.[29,30] In contrast, human EBA studies are thought to measure effects on bacteria originating predominantly from cavities, i.e. necrotic tissue with mostly extracellular organisms.[21]

Thirdly, the reproducible onset of SQ109 action in murine TB is after 3–4 weeks.[9,11] Administration of SQ109 in combination with other agents over a longer period might allow the efficacy of this agent to manifest.

In summary, this randomized controlled Phase 2a study showed that SQ109 is reasonably tolerated and safe in patients with pulmonary TB at doses of 75, 150 and 300 mg alone and combined with rifampicin. Plasma concentrations of SQ109 remained below the MICs. No bactericidal effect of SQ109 alone or enhancement of rifampicin activity in 2 weeks of treatment was seen. The dose of 300 mg showed the highest exposures in plasma and seemed to compensate for interaction with rifampicin, which was observed with the dose of 150 mg of SQ109. SQ109 did not affect exposure to rifampicin.

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Transparency declarations

C. A. N. is the founder and CEO of Sequella, Inc. G. H. and A. J. P. are Sequella, Inc. employees. C. A. N., G. H. and A. J. P. own Sequella, Inc. stock. All other authors: none to declare. The design of this study was discussed with Sequella, Inc. The funders of this study did not play any other decision-making role in the execution, analysis or reporting of the research.

Author contributions

Study conception and design: N. H., M. H., G. H., C. A. N. and A. H. D. Study and laboratory analyses: J. d. B., R. D., K. N., A. V., R. E. A., A. H. D. and S. H. Data analysis: N. H., R. E. A., M. H., P. P. J. P. and A. H. D. Manuscript drafted for important intellectual content: N. H., C. A. N., A. J. P., R. E. A., M. J. B., S. H. G., A. R., P. P. J. P., M. H. and A. H. D. Sequella, Inc. authors contributed to the Introduction and Discussion sections. Final decision on publication and content: M. H., N. H. and A. H. D.

Supplementary data

Figure S1 is available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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Publikation 5:

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High seroprevalence for spotted Fever group rickettsiae, is associated with higher temperatures and rural environment in Mbeya region, southwestern Tanzania.

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RESEARCH ARTICLE

High Seroprevalence for Spotted Fever Group Rickettsiae, Is Associated with Higher Temperatures and Rural Environment in Mbeya Region, Southwestern Tanzania

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Abstract

Background

Rickettsioses are endemic in sub-Sahara Africa. Burden of disease, risk factors and transmission are hitherto sparsely described.

Methods

From the EMINI (Evaluating and Monitoring the Impact of New Interventions) population cohort, we randomly selected 1,228 persons above the age of 5 years from the nine participating communities in Mbeya region, Southwestern Tanzania, stratified by age, altitude of residence and ownership of domestic mammals, to conduct a cross-sectional seroprevalence study in. The aim was to estimate the seroprevalence of IgG antibodies against Spotted Fever Group (SFG) rickettsiae and to assess socioeconomic and environmental risk factors. Serology (indirect immunofluorescence) was performed at a dilution of 1:64.

Results

SFG-seropositivity in the cohort was found to be 67.9% (range among nine sites: 42.8–91.4%). Multivariable analysis revealed an association with age (prevalence ratio, PR per 10 years: 1.08; 95% CI 1.06–1.10), warmer temperatures (PR per °C: 1.38; 1.11–1.71), male gender (PR 1.08; 1.00–1.16), and low population density (PR per 1.000 persons/km² increase 0.96; 0.94–0.99). At higher elevations, higher cattle density was associated with higher seroprevalence.

Conclusion

SFG rickettsial infection seems to be common in the more rural population of Mbeya Region. Spread seems to be further limited by temperature and higher elevation. Examination of the contribution of SFG to febrile illnesses seems warranted in a prospective study to estimate the disease burden in the population. This will also allow determination of the causative pathogens.

Author Summary

We report a high seroprevalence for antibodies against Spotted Fever Group (SFG) rickettsiae in Southwestern Tanzania, a group of bacteria that is mostly transmitted by ectoparasites such as fleas, lice, mites, and ticks. Serum samples from 1.228 persons were selected, and 67.9% were positive indicating past infection. Seropositivity was clearly associated with warmer temperature, low population density and elevations below 1,500 m. These infections may have been caused by *Rickettsia africae*, which is transmitted to humans by a cattle tick, and was found in similar settings in Africa. To investigate further on the significance of SFG rickettsioses on disease burden in the area, investigations in acutely febrile patients should be conducted.

Introduction

Tick-borne rickettsiae were first detected in the early 20th century, and have frequently been reported from all over the world including the Mediterranean and sub-Sahara African countries. Until recently all spotted fever cases in Africa were suspected to be Mediterranean Spotted Fever (MSF), caused by the obligatory intracellular bacterium *Rickettsia conorii*. In 1996, Kelly et al. identified the new species *R. africae* as the pathogenic agent of African Tick Bite Fever (ATBF) and as part of the Spotted Fever Group (SFG) rickettsiae [1]. Therefore Pijper's suggestion from 1936 to differentiate between spotted fevers with a more and less severe prognosis [2] could be fortified, with ATBF falling into the latter group [3].

In travel medicine, tick-borne rickettsioses are regarded as the second most frequently diagnosed tropical disease entity in febrile patients returning from rural sub-Sahara Africa [4,5]. High rates of antibody seropositivity against SFG rickettsiae have been reported for the populations of many African countries such as Angola, Burkina Faso, the Central African Republic, the Ivory Coast, Congo, Mali [6], Kenya [7], Mauritania [8], Zambia [9], Zimbabwe [10,11] and most recently Senegal [12]. In northern Tanzania, 8% of acutely febrile hospitalized patients were serologically diagnosed with SFG rickettsia infections [13]. In contrast, a study on febrile pediatric outpatients <10 years from western and central Tanzania found a rickettsial cause in only 1% (10 of 1005); with six children diagnosed with typhus group, and four with a SFG rickettsial infection by serology. While the epidemiological importance of SFG rickettsioses in Africa is increasingly recognised, only few data are available on the distribution of SFG rickettsial species, the burden and the severity of disease, geographic localisation and on risk factors for acquiring infection, which could probably help in understanding the differences between the two Tanzanian cohorts mentioned. Consequently, misdiagnosis and mistreatment are frequent and preventive measures are rare.

The typical clinical triad in rickettsiosis consists of a maculopapular rash, fever and an eschar, but the occurrence of symptoms and the prognosis vary between the different types of spotted fevers [12]. Pathogens like *R. conorii* and *R. massiliae* seem to be related to a more severe disease, while ATBF, which is caused by *R. africae*, and presents with fever and often multiple eschars, is a benign disease. Vectors and reservoir hosts differ between SFG species. While *R. africae* is transmitted mostly by the cattle ticks *Amblyomma hebraeum* or *variegatum*, [12], *R. conorii* and *R. massiliae* are found predominantly in the brown dog tick *Rhipicephalus sanguineus* and disease caused by those agents is more likely to be contracted in urban areas [2].

Methods

The following description of methods and population were already included in previously published reports with serostudies on different infectious agents; except for the serological method employed here [14–16].

Ethics

Both EMINI and this sub-study were approved by Mbeya Medical Research and Ethics Committee, and the Tanzanian national Medical Research Coordinating Committee. Each EMINI participant had provided written informed consent before enrolment. Parents consented for participation of their minor children. Data and samples were anonymized using an alphanumeric code. Linkage to personal identifying information was only possible via a key database to which only the head of data management at the Tanzanian site had access.

Study population

The EMINI study was a population-based survey with longitudinal follow up, created for providing the basis to **E**valuate and **M**onitor the **I**mpact of **N**ew **I**nterventions in the Mbeya Region of Southwestern Tanzania.

Data and samples for this study were collected between June 2007 and June 2008 during the second annual survey of the EMINI cohort study. Before the start of EMINI, a census of the complete population had been conducted in nine geographically distinct sites of the Mbeya Region of Southwestern Tanzania, which had been selected to represent a wide variety of environmental and infrastructural settings including urban and rural sites, different proximity to main roads and elevation above sea-level. During the census we collected basic information regarding the households and their inhabitants, and recorded all household positions, using handheld GPS receivers. Ten percent of the census households and all their inhabitants were chosen by geographically stratified random selection to participate in the 5-year longitudinal EMINI cohort study, resulting in a representative sample of the population in the nine study sites. Every year, each participating household was visited to conduct structured interviews and to collect blood and other specimen from all household members. Blood samples were cryopreserved after cells were separated from serum.

For this sub-study, we stratified the 17,872 participants, who had provided a blood sample in the second EMINI survey, by their age, altitude of residence and ownership of domestic mammals, to be able to assess factors of interest that had been identified in the literature but might have been underrepresented in the general population. We employed disproportionate random sampling with equal participant numbers for each stratum to identify samples from 1228 participants above the age of 5 years to be tested for IgG antibodies against Spotted Fever Group (SFG) rickettsiae using *R.conorii* as surrogate antigen.

Socio-economic status

During the annual EMINI visits, we conducted interviews with the head of each household regarding the socio-economical and infrastructural setting in and around the household. With this information we constructed an SES score to characterize the socio-economic situation of each household, employing a modification of a method originally proposed by Filmer and Pritchett that uses principal component analysis and has been widely applied to assess wealth and poverty in developing countries [17–19]. The score included the following information: Availability of different items in the household (clock or watch, radio, television, mobile telephone, refrigerator, hand cart, bicycle, motor cycle, car, savings account); sources of energy and drinking water; materials used to build the main house; number of persons per room in the household and availability and type of latrine used.

Environmental data

Population- and livestock-densities were calculated using data and household positions collected during the initial population census. Elevation data were retrieved from the NASA Shuttle Radar Topography Mission (SRTM) global digital elevation model, version 2.1 [20,21]. Land surface temperature (LST) and vegetation cover (EVI = enhanced vegetation index) data for the years 2003 through 2008 were retrieved from NASA's Moderate-resolution Imaging Spectroradiometer (MODIS) mission which "are distributed by the Land Processes Distributed Active Archive Center (LP DAAC), located at the U.S. Geological Survey (USGS) Earth Resources Observation and Science (EROS) Center (lpdaac.usgs.gov)." [22]. These data were used to produce long-term averages of day and night LST and EVI. Population-, household-, and livestock-densities, LST, EVI, and elevation data were averaged for a buffer area within 1000 meter radius around each household in order to characterize the ecological situation around the household. This approach was preferred to using the respective spot values at the household position, because spot data are more prone to random error than averages for a wider area.

Serology

Serum samples were tested for antibodies against SFG-Rickettsia by indirect immunofluorescence (IIFA) with a commercially available test (Rickettsia conorii Spot IF, Fuller Laboratories, Fullerton, U.S.A.). Serum samples were used at a dilution of 1:64, and developed using a polyclonal rabbit anti-human IgG immunoglobin labelled with fluorescein isothyocyanate. All slides were independently examined by two experienced laboratory workers. In case of discrepancies results were discussed and slides compared to positive controls until agreement was reached.

Commercial negative and positive controls were used in each test. Fluorescence of the rickettsiae with an intracellular distribution and intensity pattern similar to the positive control was considered as a positive reaction. Titres of 64 or higher were considered sero-reactive and further titration of the sera was not done.

Data analysis

Stata statistics software (version 13, StataCorp., College Station, TX, USA) was used for all statistical analyses, and Manifold System 8.0 Professional Edition (Manifold Net Ltd, Carson City, NV) was used for processing of geographical data and to produce maps. In order to identify possible risk factors for SFG IgG positivity, we analysed seropositivity as the binary outcome in uni- and multi-variable Poisson regression models with robust (or Huber-White) variance estimates adjusted for household clustering [23,24]. Initial uni-variable models for all factors that

we deemed as possibly related to SFG infection were used to identify variables with a uni-variable p-value < = 0.1 for further multi-variable evaluation. The following variables were included into multi-variable evaluation without consideration of their p-values: age and gender (as basic confounders), and the two cattle related variables (because of the assumed role of cattle in the transmission of SFG). Continuous variables were categorized into quintiles and both, representations of the data examined. If possible, we used the continuous variable, but if the categorical representation of the variable showed strong evidence of a nonlinear association with SFG IgG positivity (e.g. a strongly concave or convex association), the categorical version was used. Stepwise backward and forward regression, the Akaike and Bayes information criterion and various assessments of model-fit were used to identify the final multi-variable model, where only variables with a multi-variable p-value <0.1 were retained. If variables were strongly collinear (which was common in the environmental variables, e.g. ambient temperature, rainfall, elevation, slope, vegetation density etc.) as witnessed by a variance inflation factor > = 5, the variable that provided the best model fit was retained in the final multi-variable model. Study site was not included into the final model because it was strongly collinear with the environmental variables. All ecological variables in the final model were tested for two-way interaction, and the interaction terms included into the model, if a significant interaction was present.

Results

Of the 1,228 analysed sera, 67.9% overall were positive for SFG IgG. Seropositivity varied considerably between the nine different sites (Table 1 and Fig 1).

Socio-economic risk factors

Seropositivity increased with age (Table 2 and Fig 2), with a prevalence of 43.6% in children between 5 and 8 years of age, the youngest age group examined.

Male participants were significantly more often seropositive than females (multi-variable analysis: prevalence ratio (PR) = 1.08, 95% confidence interval (CI) = 1.00 to 1.16). Seropositivity was higher in areas with low population density (PR = 0.93 per 1000/km^2, CI = 0.90 to 0.96). An association of SFG IgG with socio-economic status, which was significant in uni-variable analysis, was rendered non-significant in the multi-variable model.

Ecological risk factors

Seropositivity was strongly associated with the annual average land surface temperature during the day (PR = 1.31 per °C, CI = 1.05 to 1.64).

Elevation is associated with SFG IgG in uni-variable and multi-variable models, but the relation was non-linear, with a significant decline above 1,578 meters.

Since elevation and rainfall could not be included into the final model simultaneously due to collinearity, rainfall was excluded since elevation was the better predictor of infection.

Cattle density showed a non-significant trend for positive association in uni-variable analysis. Multi-variable interaction analysis with elevation showed cattle density to be positively associated with seropositivity only in higher elevation strata above 1291 m. The association with number of cattle owned was rendered non-significant in the multi-variable model, suggesting that the local density of animals is more important than ownership. Other suspected risk factors seemed to have no significant impact such as the possession of goats (PR in uni-variable analysis 1.01, 95% CI 0.99–1.03, p = 0.215), pigs (PR 0.97, 95% CI 0.88–1.07; p = 0.064), chicken (PR 1.04, 95% CI 0.95–1.14, p = 0.353), or dogs (PR 1.02, 95% CI 0.91–1.14, p = 0.750).

Table 1. Characteristics of the Study Population and their places of residence.

	Igurusi (N = 204)	Mlowo (N = 105)	Santylia (N = 85)	Isongole (N = 138)	Itaka (N = 110)	Utengule (N = 170)	Ruanda (N = 97)	Kyela (N = 229)	Iyunga (N = 90)	Total (N = 1228)
SFG seroprevalence	81.4%	91.4%	51.8%	42.8%	82.7%	77.6%	45.4%	70.7%	44.4%	67.9%
Age[a]	38.3	33.5	36.2	37.0	28.5	35.6	33.9	29.5	36.7	33.9
IQR	21.8 to 53.3	17.2 to 46.1	20.4 to 51.2	15.9 to 56.4	13.9 to 45.0	18.6 to 54.3	16.5 to 51.9	15.5 to 50.8	17.6 to 53.9	17.1 to 52.0
Socio-Economic-Status Score[a]	-0.19	0.29	-0.49	-0.42	-0.22	-0.20	1.17	-0.55	0.50	-0.14
IQR	-0.65 to 0.48	-0.03 to 0.80	-0.81 to -0.04	-0.77 to 0.18	-0.64 to 0.26	-0.59 to 0.36	0.71 to 1.79	-1.02 to 0.06	0.14 to 1.86	-0.64 to 0.52
Population Density (/km^2) [a]	1,541	4,022	205	626	216	217	11,788	471	1.513	461
IQR	284 to 2,173	223 to 4780	139 to 291	337 to 888	150 to 278	95 to 286	11,547 to 12,069	344 to 731	1,070 to 2,187	223 to 1,956
Cattle (/km^2) [a]	163	265	33	24	42	37	252	169	70	81
IQR	54 to 205	76 to 287	21 to 44	11 to 72	30 to 73	27 to 50	244 to 258	140 to 186	54 to 81	37 to 187
Distance to main road (km) [a]	0.4	0.8	16.6	0.3	29.0	5.0	0.2	3.9	0.7	2.2
IQR	0.2 to 0.9	0.5 to 1.1	14.7 to 18.8	0.13 to 0.95	26.9 to 30.9	3.4 to 10.9	0.12 to 0.32	2.5 to 6.0	0.3 to 1.2	0.4 to 6.2
Elevation (m) [a]	1,193	1,580	2,018	2,009	1,509	1,346	1,714	487	1,604	1,491
IQR	1,155 to 1,205	1,575 to 1,585	1,985 to 2,068	1,892 to 2,223	1,477 to 1,554	1,274 to 1,449	1,711 to 1,728	483 to 514	1,591 to 1,619	1,157 to 1,710
max. EVI [a]	0.50	0.45	0.54	0.49	0.42	0.53	0.26	0.53	0.47	0.50
IQR	0.49 to 0.51	0.43 to 0.47	0.52 to 0.54	0.48 to 0.53	0.41 to 0.44	0.52 to 0.55	0.25 to 0.28	0.50 to 0.56	0.44 to 0.49	0.45 to 0.53
Avg. Land Surface Temp. (°C) [a]	34.6	33.6	29.9	28.0	34.3	33.0	31.9	32.3	33.5	33.3
IQR	34.2 to 35.7	33.3 to 33.7	29.2 to 30.8	26.1 to 29.0	34.1 to 34.6	31.1 to 34.0	31.6 to 32.6	30.8 to 33.5	33.3 to 33.7	30.8 to 34.1

a: medians for respective sites

IQR = interquartile range

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Discussion

Our data show that lifetime risk of SFG infection in the study area is very high, with seroprevalences up to 80% in higher age strata. Many infections already seem to occur in early childhood below the age of 5 years.

Several socio-economic and environmental factors seem to play a role in infection. Higher temperatures as one of the stronger risk factor are biologically plausible, since those seem to increase host-seeking behaviour in many ticks [25,26], among other influences on tick breeding and survival.

Further, our analyses find associations with lower population density in multi-variable analysis, thus incidence seems higher in rural communities. Population itself may not be the only driving factor here: in our setting this variable and vegetation density are inversely collinear; thus one of both factors usually drops out of multi-variable analysis, with limited certainty on which factor is a better predictor of SFG IgG seropositivity [14]. Vegetation is a proxy for water and humidity, so a denser vegetation could mean a more humid environment; dryness

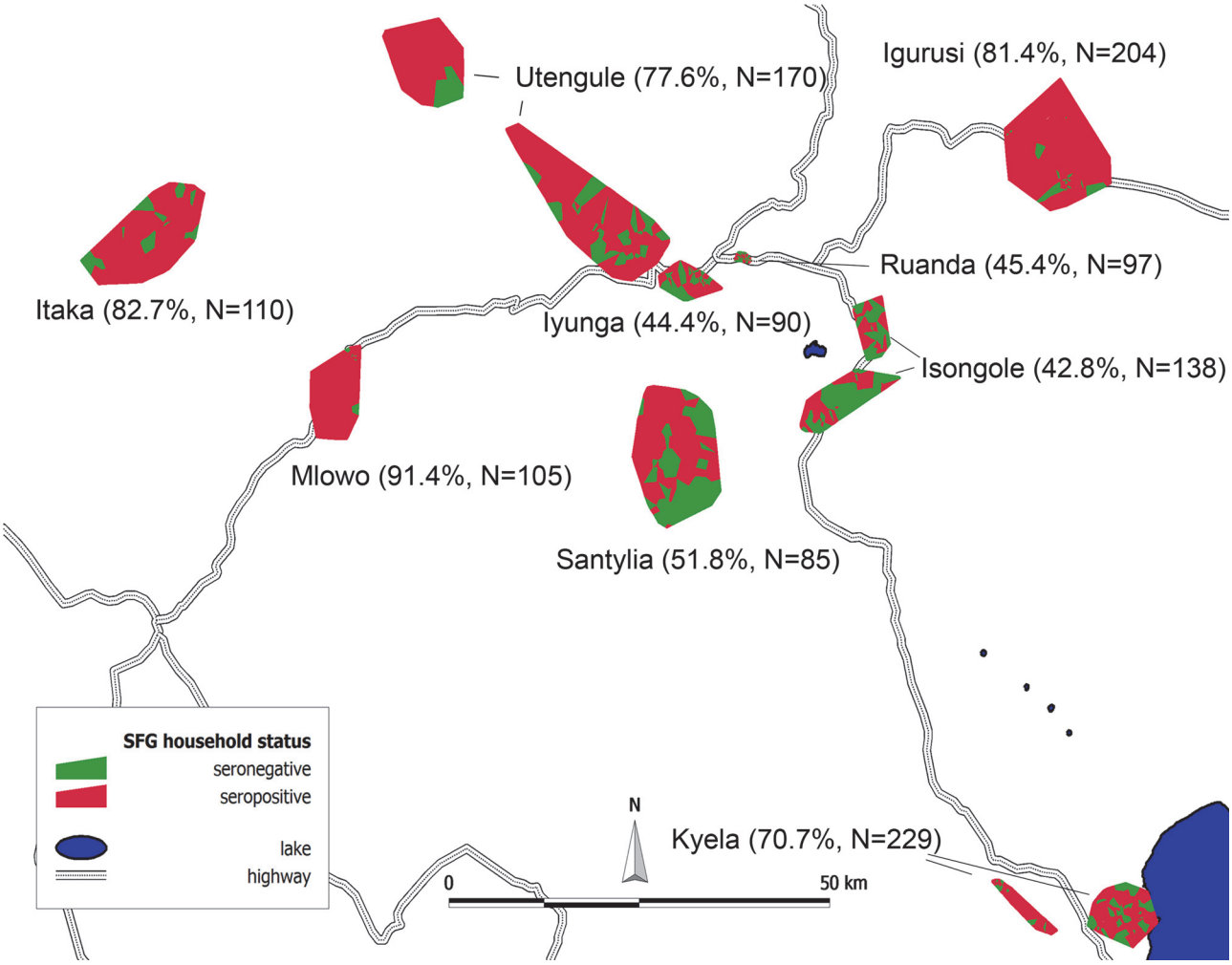

Fig 1. Location of households with SFG IgG-positive participants displayed as Voronoi polygons, with every polygon representing one household.

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reportedly affects tick host-seeking behaviour in several tick species [25,26]. Comparing these data to our published analysis of typhus group rickettsial (TGR) antibodies, it is evident that TGR and SFG seropositivity occur in different geographical patterns, with higher prevalence in areas with sparse vegetation; or dense population for TGR [14]. TGR in our area probably rely on a rat reservoir, which is probably more abundant in cities. The likely vector is *Xenopsylla cheopsis*, the rat flea, which may be less dependent on elevation, outer temperature and humidity than the SFG vector due to more continuous proximity to its vertebrate host.

The non-linear association of SFG seropositivity with elevation could be due to two independent effects. High elevations with their colder and rougher conditions are likely to negatively affect the tick vector carrying the pathogen. The lowest elevation stratum (below 974 m) with lower seroprevalence, consists of one site, Kyela. This site has abundant collections of surface water, and may thus be unsuitable to tick development despite it' s favourable warm and moist climate. Contrarily to SFG, the mosquito-borne diseases like Rift Valley fever or Alphavirus show the highest seroprevalences in this site, which is probably due the availability of surface water [15,16].

Table 2. Socio-demographic and environmental factors and their association with Spotted Fever Group Rickettsial Seropositivity (N = 1228).

Covariate	stratum	N[a]	uni-variable				Multi-variable		
			% pos.[b]	PR[c]	95% CI[d]	p-val	PR[c]	95% CI[d]	p-val
Age									
	per 10 years	1228	67.9	1.07	(1.05 to 1.09)	<0.001	1.08	(1.06 to 1.10)	<0.001
Gender									
	female	674	66.2	1	-	-	1	-	-
	male	546	70.0	1.06	(0.98 to 1.14)	0.160	1.08	(1.00 to 1.16)	0.040
	miss.	8	75.00	1.13	(0.76 to 1.70)	0.544	1.15	(0.82 to 1.63)	0.414
Persons/km^2									
	per 1000 persons	1228	67.9	0.97	(0.96 to 0.99)	0.001	0.93	(0.90 to 0.96)	<0.001
Average Land Surface Temperature									
	per unit	1228	67.9	1.93	(1.63 to 2.28)	<0.001	1.31	(1.05 to 1.64)	0.016
Elevation (meters)									
	479-	245	71.8	1	-	-	1	-	-
	974-	246	80.9	1.13	(1.02 to 1.25)	0.022	1.00	(0.80 to 1.26)	0.971
	1291-	245	81.6	1.14	(1.03 to 1.26)	0.013	0.95	(0.76 to 1.19)	0.656
	1578-	246	58.9	0.82	(0.72 to 0.94)	0.004	0.66	(0.51 to 0.87)	0.003
	> = 1725	246	46.3	0.65	(0.55 to 0.76)	<0.001	0.59	(0.43 to 0.80)	0.001
Interaction of Elevation with Cattle/km^2									
	479-						1	-	-
	974-						1.05	(0.91 to 1.21)	0.516
	1291-						1.21	(1.04 to 1.39)	0.011
	1578-						1.35	(1.14 to 1.59)	<0.001
	> = 1725						1.41	(1.09 to 1.82)	0.008
Cattle/km^2									
	per 100 animals	1228	67.9	1.03	(0.99 to 1.08)	0.145	1.00	(0.88 to 1.13)	0.995
SES score[e]									
	per unit	1228	67.9	0.95	(0.91 to 0.99)	0.013	*This and the following variables were not included into the multi-variable model due to lack of multi-variable significance*		
Number of cattle owned									
	per animal	1228	67.9	1.01	(1.01 to 1.02)	<0.001			
Number of goats owned									
	per animal	1228	67.9	1.01	(0.99 to 1.03)	0.215			
Distance to nearest highway									
	per km	1228	67.9	1.01	(1.00 to 1.01)	<0.001			
Rainfall (in mm)									
	101.4-	235	63.4	1	-	-			
	114.6-	227	70.5	1.11	(0.97 to 1.27)	0.114			
	123.9-	274	84.3	1.33	(1.19 to 1.49)	<0.001			
	147.2-	246	55.3	0.87	(0.75 to 1.02)	0.079			
	192.4-	246	64.2	1.01	(0.88 to 1.16)	0.855			
Enhanced vegetation index (max.)									
	per 0.1 units	1228	67.9	1.09	(1.03 to 1.15)	0.004			
Slope									
	per degree	1228	67.9	0.98	(0.96 to 1.00)	0.015			

Results of poisson regression analysis with robust variance estimates adjusted for household clustering.

a: Number of participants in each stratum (stratified variables only)

b: percent sero-positive in stratum

c: Prevalence ratio

d: 95% confidence interval

e: Socio-Economic-status according to socio economic score

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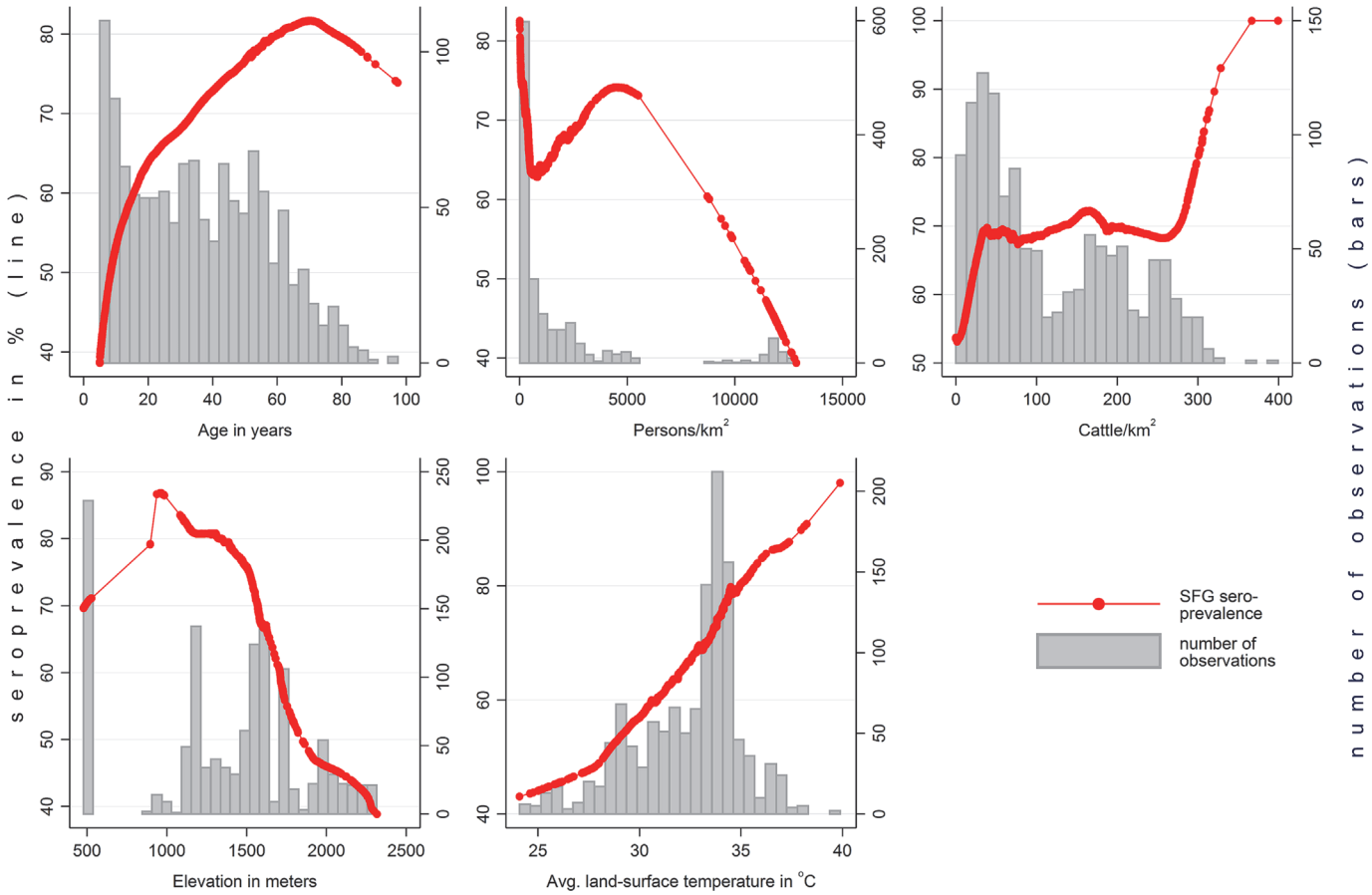

Fig 2. Lowess smoothed plots of SFG-seroposivitivity over age, population density, cattle density, temperature and elevation. Thin lines represent extrapolations, i.e. areas without data.

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The predominant SFG species remains to be established. The association of seropositivity with cattle (which is evident in higher elevation strata above 974 m) allows speculations whether a cattle parasite could be involved as vector, and/or cattle could play a role as a reservoir host for the pathogen. A countrywide survey of cattle ticks in Tanzania by others identified *Amblyomma variegatum*, the vector of *R. africae*, as the predominant cattle tick [27]. Further, high rates of SFG seropositivity were described in cattle from Zimbabwe, a country where *A. variegatum* is also endemic [11,28].

Preliminary results from our group, examining ticks collected from the study area by PCR, found 6 out of 10 *A. variegatum* ticks were positive for *R. africae*. *R.massiliae* was found in 2 out of 7 *Rhipicephalus sanguineus* and in one out of 2 *Heamaphysalis elliptica* ticks (*G. Dobler, manuscript in preparation)*. All three *Rickettsia* species found are known to be pathogenic for humans, so although *R. africae* may be the predominant SFG pathogen, others could play a role.

However, SFG rickettsiae, transmitted by the brown dog tick *Rh. sanguineus*, like *R. massiliae* or *R. conorii*, could also be the cause of the observed antibody prevalence; but those would be expected to be more frequent in urban settings [29], which does not correspond to our findings on population density.

In Tanzanian agriculture, most cattle herding is done by male family members, thus the slightly higher seroprevalence in male participants may corresponds to cattle contact as a risk factor.

Our analysis may be limited by the fact that we did not take into account the "site" variable. In EMINI, sites were specifically chosen to represent different socio-economic and ecological variables, thus the variable "site" is clearly collinear with most variables tested in this analysis. Including the variable "site" into the analysis would have implied that these variables, e.g. elevation or population density would have been corrected for and their correlation with SFG IgG rendered insignificant. This would probably have been inadequate and have led to most likely false observations of no correlation between SFG IgG and those variables. However, hypothetical variables inherent to the "site" that we were not able to capture and that may have influenced the site' s SFG IgG prevalence might thus make the association of SFG IgG to the variables analysed stronger than adequate.

In conclusion, our findings are compatible with previous descriptions and add information, especially on risk factors for SFG rickettsioses in southwestern Tanzania. SFG might thus contribute to disease burden—other studies describe SFG rickettsioses to be frequent in travellers from Africa, and as endemic diseases in African countries [4,5,12,30]. Future studies should aim to detect the pathogen in acute infection, and to describe the local transmission cycle in order to validate the identified risk factors in a prospective way.

Supporting Information

S1 Checklist. STROBE checklist.
(DOC)

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Author Contributions

Conceived and designed the experiments: NH GD MH ES TL PC LM. Performed the experiments: TD NEN IK EN PC. Analyzed the data: NH TD ES MH. Contributed reagents/materials/analysis tools: GD. Wrote the paper: NH TD GD ES IK PC EN LM MH TL MS.

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PLoS Negl Trop Dis. 2012;6(3):e1557.

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PLoS NEGLECTED TROPICAL DISEASES

High Seroprevalence of Rift Valley Fever and Evidence for Endemic Circulation in Mbeya Region, Tanzania, in a Cross-Sectional Study

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Abstract

Background: The Rift Valley fever virus (RVFV) is an arthropod-borne phlebovirus. RVFV mostly causes outbreaks among domestic ruminants with a major economic impact. Human infections are associated with these events, with a fatality rate of 0.5–2%. Since the virus is able to use many mosquito species of temperate climates as vectors, it has a high potential to spread to outside Africa.

Methodology/Principal Findings: We conducted a stratified, cross-sectional sero-prevalence survey in 1228 participants from Mbeya region, southwestern Tanzania. Samples were selected from 17,872 persons who took part in a cohort study in 2007 and 2008. RVFV IgG status was determined by indirect immunofluorescence. Possible risk factors were analyzed using uni- and multi-variable Poisson regression models. We found a unique local maximum of RVFV IgG prevalence of 29.3% in a study site close to Lake Malawi (N = 150). The overall seroprevalence was 5.2%. Seropositivity was significantly associated with higher age, lower socio-economic status, ownership of cattle and decreased with distance to Lake Malawi. A high vegetation density, higher minimum and lower maximum temperatures were found to be associated with RVFV IgG positivity. Altitude of residence, especially on a small scale in the high-prevalence area was strongly correlated (PR 0.87 per meter, 95% CI = 0.80–0.94). Abundant surface water collections are present in the lower areas of the high-prevalence site. RVF has not been diagnosed clinically, nor an outbreak detected in the high-prevalence area.

Conclusions: RVFV is probably circulating endemically in the region. The presence of cattle, dense vegetation and temperate conditions favour mosquito propagation and virus replication in the vector and seem to play major roles in virus transmission and circulation. The environmental risk-factors that we identified could serve to more exactly determine areas at risk for RVFV endemicity.

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Introduction

The Rift Valley fever virus (RVFV), a member of the genus Phlebovirus in the family Bunyaviridae, was first isolated in 1930 during an outbreak in Kenya. Rift Valley fever (RVF) occurs endemically and epidemically in most parts of sub-Saharan Africa and epidemically in Egypt, Madagascar and the Comoros. In 2001 it was detected for the first time outside of Africa during an outbreak in Yemen and Saudi-Arabia [1,2,3,4,5].

The disease is mostly apparent in epizootic events with large numbers of sick cattle, and a high abortion rate in pregnant animals (“abortion storm”), with adverse economic consequences for cattle herders, including bans on animal trade [4]. Transmission to humans is common during such events. In the majority of cases, human infection is oligo- or asymptomatic, but may cause hepatitis, hemorrhagic fever, encephalitis and retinitis, with fatality rates of 0.5 to 2%, and permanent vision impairments after retinitis [4].

Contrary to the assumption of virus persistence and inactivity between outbreaks, some evidence for inter-epidemic circulation of RVFV has been reported from the Senegal and from northern Kenya, using a serology approach to detect antibodies in samples from children born after the last reported outbreak [6,7].

The most important vectors for RVFV are *Aedes* and *Culex* mosquitoes. However, RVFV has also been isolated from *Anopheles spp*, *Simulium* blackflies, sand flies and *Amblyomma* ticks [2,4,8], which may represent remnants of a blood meal rather than the

Author Summary

We describe a high seropositivity rate for Rift Valley fever virus, in up to 29.3% of tested individuals from the shore of Lake Malawi in southwestern Tanzania, and much lower rates from areas distant to the lake. Rift Valley fever disease or outbreaks have not been observed there in the past, which suggests that the virus is circulating under locally favorable conditions and is either a non-pathogenic strain, or that occasional occurrence of disease is missed. We were able to identify a low socio-economic status and cattle ownership as possible socio-economic risk factors for an individual to be seropositive. Environmental risk factors associated with seropositivity include dense vegetation, and ambient land surface temperatures which may be important for breeding success of the mosquitoes which transmit Rift Valley fever, and for efficient multiplication of the virus in the mosquito. Low elevation of the home, and proximity to Lake Malawi probably lead to abundant surface water collections, which serve as breeding places for mosquitoes. These findings will inform patient care in the areas close to Lake Malawi, and may help to design models which predict low-level virus circulation.

ability to transmit the pathogen. Direct transmission through infectious body fluids is of relevance mainly during epizootic/epidemic events [5,9]. As many competent vector species occur outside Africa, a high potential for further geographical spread is attributed to the virus, and RVF is classified as an emerging disease [4,10].

RVF outbreaks are known to occur predominantly after unusual flooding events. *Aedes* mosquito species are seen as vectors and reservoir, since their transovarially infected eggs withstand desiccation and larvae hatch when in contact with water [6,11]. Transovarial transmission is assumed as mechanism of virus persistence between epizootic events.

After flooding, the *Aedes* mosquito populations will multiply in the persisting water collections, and develop into infectious adult mosquitoes. The RVFV may amplify in wild and domestic ungulates and may reach epizootic and epidemic dimensions [8]. The presumed link between extraordinary flooding events and RVF outbreaks was validated, among others, by a successful prediction of the 2007 outbreak in Somalia, Kenya and northern Tanzania, using climate modelling [12].

A number of variables associated with higher likelihood for RVFV Immunoglobulin G (RVFV IgG) positivity have been identified. Among them are the proximity to perennial surface water bodies and proximity to ruminants [7,13].

Here we report a cross-sectional seroprevalence study that used samples from 1228 participants collected during a cohort study (EMINI) from the Mbeya region in Southwestern Tanzania, an area from which no RVF disease activity has been reported previously. The objective was to assess any RVFV circulation that had possibly remained undetected, and to describe infection patterns and factors associated with seropositivity.

Methods

Ethics statement

Both EMINI and this substudy were approved by Mbeya Medical Research and Ethics Committee, Tanzanian National Institute for Medical Research – Medical Research Coordinating Committee, as well as by the Ethical Commission of University of Munich. Each EMINI participant had provided written informed consent before enrolment. Parents consented for participation of their children.

Study population

Data and samples for this study were collected between June 2007 and June 2008 during the second annual survey of the EMINI (Evaluating and Monitoring the Impact of New Interventions) cohort study. Before the start of EMINI, a census of the complete population had been conducted in nine geographically distinct sites of the Mbeya Region in Southwestern Tanzania, which had been selected to represent a wide variety of environmental and infrastructural settings, including urban and rural sites, different proximity to main roads, elevation above sea-level etc (Figure 1). During the census we collected basic information regarding the households and their inhabitants, and recorded all household positions, using handheld GPS receivers. Ten percent of the census households and all their inhabitants were chosen by geographically stratified random selection to participate in the 5-year longitudinal EMINI cohort study, resulting in a representative sample of the population in the nine study sites. Every year, each participating household was visited to conduct structured interviews and to collect blood and other specimen from all household members. Blood samples were cryopreserved after cells were separated from serum.

For this substudy, we stratified the 17,872 participants, who had provided a blood sample in the second EMINI survey, by age, gender, altitude of residence and ownership of domestic animals (mammals), to be able to assess factors of interest that were identified in the literature but might have been underrepresented in the general population. We employed disproportionate random sampling with equal participant numbers for each stratum to identify 1228 samples from participants above the age of 5 years to be tested for RVFV IgG.

Socio-economic status (SES)

During the annual EMINI visits, we conducted interviews with the head of each household regarding the socio-economical and infrastructural setting in and around the household. With this information we constructed an SES score that characterizes the socio-economic situation of each household, employing a modification of a method originally proposed by Filmer & Pritchett that uses principal component analysis and has been widely applied to assess wealth and poverty in developing countries [14,15,16]. The score included the following information: Availability of different items in the household (clock or watch, radio, television, mobile telephone, refrigerator, hand cart, bicycle, motor cycle, car, savings account); sources of energy and drinking water; materials used to build the main house; number of persons per room in the household and availability and type of latrine used.

Environmental data

Population- and livestock-densities were calculated using data and household positions collected during the initial population census.

Elevation data were retrieved from the NASA Shuttle Radar Topography Mission (SRTM) global digital elevation model, version 2.1 [17,18].

Land surface temperature (LST) and vegetation cover (EVI = enhanced vegetation index) data were retrieved from NASA's Moderate-resolution Imaging Spectroradiometer (MODIS) Terra mission which "are distributed by the Land Processes Distributed Active Archive Center (LP DAAC), located at the U.S. Geological Survey (USGS) Earth Resources Observation and Science (EROS)

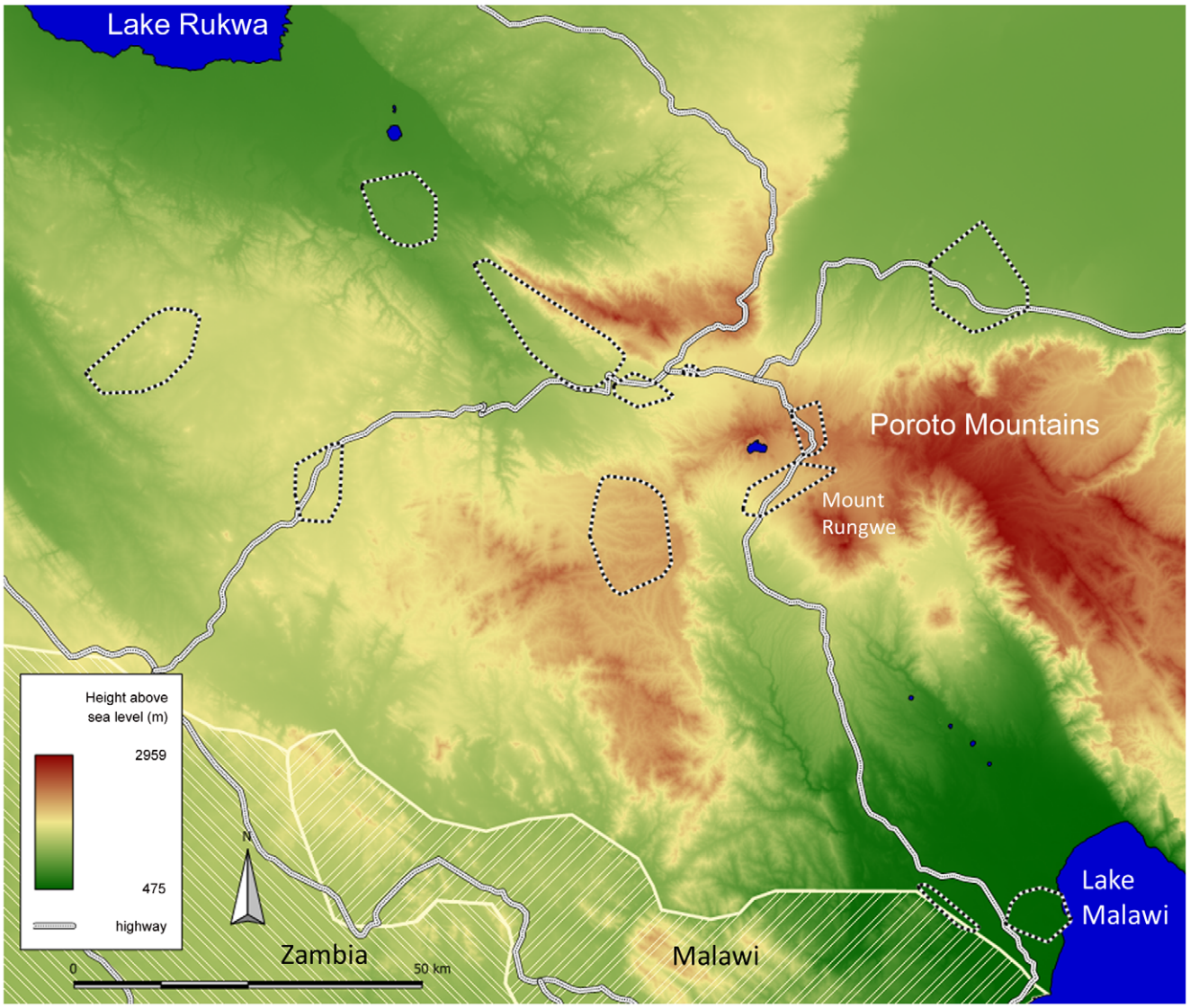

Figure 1. Location and Elevation of the Study Sites. A geographical map of the study area and sites.
doi:10.1371/journal.pntd.0001557.g001

Center (lpdaac.usgs.gov)." [19]. LST data (Version MOD11A2) have 8 days temporal and ~1 km spatial resolution , EVI data (Version MOD13Q1) have 16 days temporal and 250 m spatial resolution. Both, LST and EVI data were processed in the following way to produce long-term averages: After download via FTP, data surfaces for every 8 day period for the years 2000 to 2008 (LST) and every 16 days period for the year 2007 (EVI) were reprojected to Universal Transverse Mercator projection (zone 36 South) using the MODIS reprojection tool (MRT) [20] and imported into Idrisi GIS software (version 32, Clark Labs, Worcester, MA, USA). In Idrisi, 8 year averages of annual average and maximum day-LST and average and minimum night-LST and 2007 EVI averages were calculated for each pixel utilising only those pixels that were "good quality" according to the quality assessment layers that are distributed together with the actual data. Then LST was converted to degrees Celsius and EVI was converted back to its native range between −1 and +1.

All above environmental data were then combined with the houshold position data in a GIS database using Manifold System 8.0 Professional Edition (Manifold Net Ltd, Carson City, NV). Population-, household-, and livestock-densities, LST, EVI, and elevation data were averaged for a buffer area within 1000 meter radius around each household in order to characterize the ecological situation around the household. This approach was preferred to using the respective spot values at the household position, because spot data are more prone to random error than averages for a wider area.

Serology

Anti-RVFV IgG was detected by indirect immunofluoerescence assay (IIFA), following a methodology adapted from Swanepoel [21]. Each serum sample was screened for the presence of anti-RVFV IgG, using a commercial biochip with a mixture of infected and non-infected Vero E6 cells on one field (positive field) and non-infected Vero E6 cells on a negative control field (Euro-immun, Lübeck, Germany).

Sensitivity and specificity of the IIFA test were tested using 20 negative sera from German blood donors and five sera positive for IgG against Sandfly Toscana virus, Sandfly Naples virus, Sandfly Sicilian virus, Puumala virus, Tahyna virus and Bunyamwera

virus. No cross reactivities with other members of the genus Phlebovirus or the viruses of other genera of the family Bunyaviridae were detected.

Serum samples were screened in a dilution of 1:10, using standard procedures for IIFA. A rabbit anti-human IgG FITC-labelled antibody (DAKO, Hamburg, Germany) was used as conjugate. A sample was classified as positive if a typical fine granular cytoplasmatic fluorescence in some groups of cells on the positive field of the biochip was detected, with no detectable fluorescing cytoplasmatic signal in the negative field. Each sample was independently assessed by two experienced observers. Results were compared and re-tested if discrepant. A part of the positive sera was re-tested by titration, and all tested sera were found to have IgG titres between 1:20 and 1:640.

Data analysis

Stata statistics software (version 11, Statacorp, College Station, TX, USA) was used for all statistical analyses, maps were produced in Manifold System 8.0 Professional Edition (Manifold Net Ltd, Carson City, NV).

After exploratory data analysis, it became clear that RVFV seroprevalence in Bujonde-Kajunjumele (BK) subsite was much higher than in all other study locations. We therefore decided to first analyse data for BK separately before trying to develop models including the data for all sites.

Since none of the continuous variables that we examined was normally distributed according to the Shapiro-Wilk and Shapiro-Francia tests for normality, the median and interquartile range (instead of mean and standard deviation) of these variables are reported to characterize the study area and population in BK and in all other sites. This is also the reason why the non-parametric Wilcoxon ranksum test was used to assess differences between BK and all other sites regarding continuous variables. Differences between sites regarding binary variables (RVF seropositivity, gender and cattle ownership) were assessed by chi square testing. The association of binary RVFV IgG status with possible risk factors was examined using uni- and multi-variable poisson regression models with robust variance estimates adjusted for within household clustering [22,23]. Uni-variable regression models were used to identify possible risk-factors for inclusion into the multi-variable model for this site. Variables with a p-value$<$0.2 in uni-variable regression and other variables that did not fulfill this criterion, but where an association with RVFV IgG seemed likely due to biological reasons (gender, and all variables related to the presence of ruminants), were further evaluated in multi-variable regression models and were retained in the final multi-variable model if their p-value was $<$0.1. Because most variables characterizing the natural environment (LST, vegetation, elevation and distance to Lake Malawi) showed strong collinearity, they were not included into the same model but entered one by one into models adjusted for the other variables that were included into the final model.

Once the final multi-variable model for BK site was identified, we used the same approach to identify a multi-variable model where data for all sites including BK were pooled. Prevalence ratios (PR) and 95% confidence intervals for covariates mentioned in the text refer to multi-variate analysis within BK site, if not mentioned otherwise.

Results

Site characteristics and seroprevalence

Of the 1228 analyzed sera, 5.2% (64 sera) were positive for RVFV IgG. This translates into an estimated overall population prevalence of 3.1% when extrapolated from our stratified sample to the underlying population of the 9 study sites.

We found a unique local maximum of 29.3% (95% confidence interval (CI) 22.2–37.3) seroprevalence in Bujonde-Kajunjumele (BK), a subsite of the Kyela site, which is situated close to Lake Malawi. The prevalence in the other sites ranged from 0.0% to 3.4% (table 1, Figure 2, 3).

We thus decided to analyze covariates within the high-prevalence setting of BK site, and to compare BK to the low-prevalence sites, in order to better understand possible causes for this marked difference.

With an altitude range of 479 to 492 meters, BK is the lowest of our study sites, while the other sites range from 499 m to 2316 m (table 1, Figure 1). The two Kyela subsites BK and Katumba-

Table 1. Characteristics of Bujonde-Kajunjumele site (N = 150) and all other sites (N = 1078).

	BK-site Median (IQR) or % (N)		All other sites Median (IQR) or % (N)		p[a]
RVF IgG positive	29.3%	(44)	1.9%	(20)	$<$0.001
Female gender	55%	(82)	55%	(590)[b]	0.885
Age (years)	32.1	(17.8 to 53.2)	34.2	(16.9 to 51.9)	0.684
SES score	−0.81	(−1.19 to −0.42)	−0.06	(−0.54 to 0.55)	$<$0.001
Cattle owned	49%	(74)	28%	(307)	$<$0.001
Elevation (meters)	484	(481 to 487)	1570	(1207 to 1714)	$<$0.001
Vegetation Density (EVI*10)	3.83	(3.60 to 4.05)	2.87	(2.56 to 3.27)	$<$0.001
Max. LST (°C)	43.1	(39.5 to 45.4)	45.7	(42.9 to 46.5)	$<$0.001
Average Day LST (°C)	30.9	(29.3 to 31.8)	32.5	(30.2 to 33.2)	$<$0.001
Min. LST (°C)	14.9	(13.5 to 15.7)	11.0	(7.3 to 11.9)	$<$0.001
Cattle Density (cows/km^2)	165	(143 to 190)	68	(34 to 186)	$<$0.001
Distance to Lake Malawi (km, BK only)	2.9	(1.4 to 5.2)			

BK = Bujonde-Kajunjumele; IQR = interquartile range; SES = socio-economic status; EVI = enhanced vegetation index; LST = land surface temperature.
[a]p-value of chi square test (for binary variables) or Wilcoxon Ranksum test (for continuous variables) for difference between BK and all other sites.
[b]gender unclear for 11 participants.
doi:10.1371/journal.pntd.0001557.t001

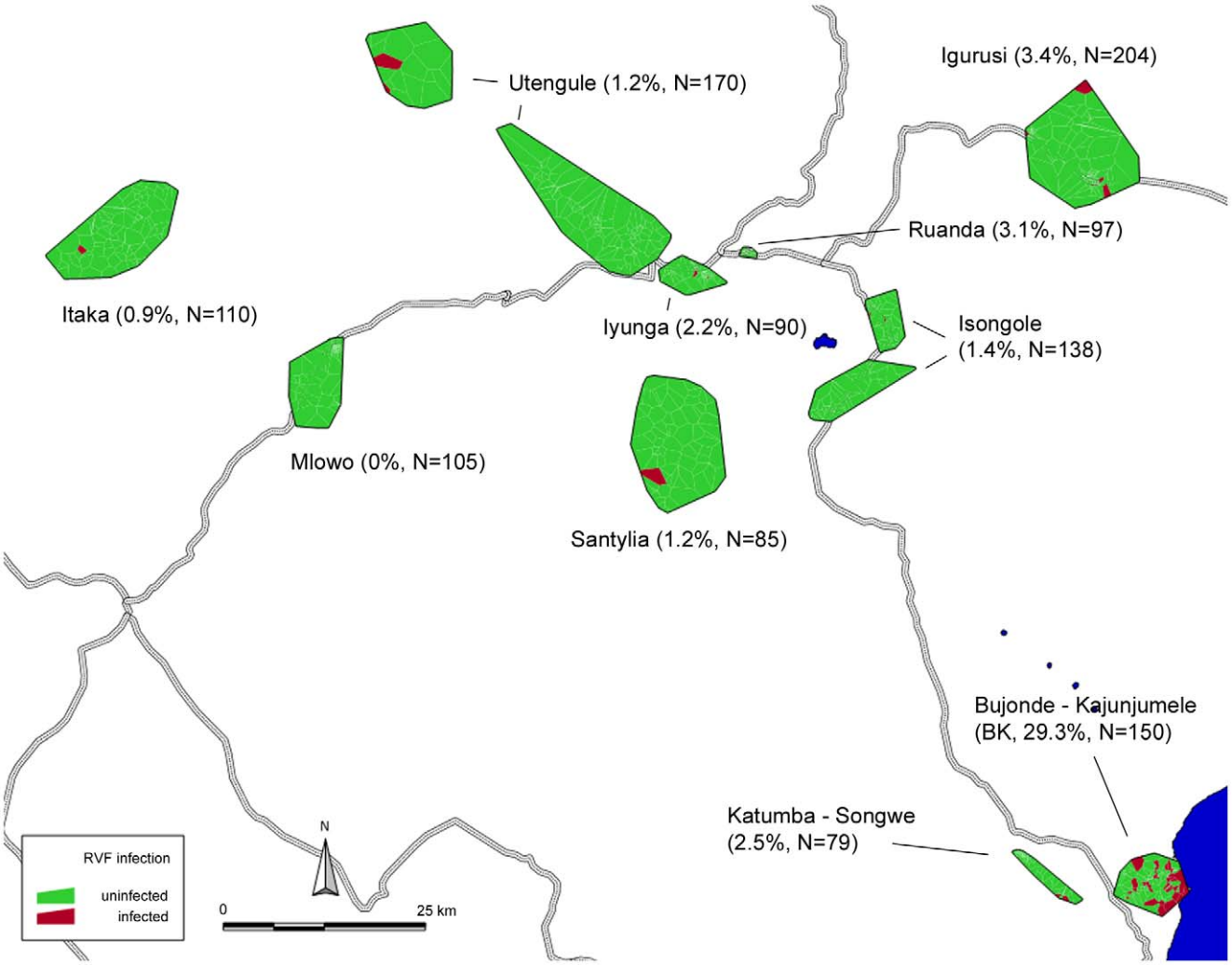

Figure 2. Location of Households with IgG-positive Participants in the entire Study Area. Location of households displayed in Voronoi polygons, with every polygon representing one household.
doi:10.1371/journal.pntd.0001557.g002

Songwe are the only sites south of the Poroto mountain range, and receive the highest amount of annual rainfall (1956 and 2292 mm, respectively), whereas the average across all sites is 1473 mm.

Further characteristics of BK site, compared to all other sites, are listed in table 1. Of special relevance are denser vegetation, lower temperature variability (higher minimum and lower maximum land surface temperatures), higher cattle density and more frequent ownership of cattle, which is presumed to be the main animal host of RVFV. During the rainy season, wide areas close to Lake Malawi are flooded, especially where the terrain is marshy and barely above the Kiwira river's water level (Figure 4).

Analysis of potential risk-factors

As demonstrated in Figure 5, RVFV IgG prevalence rises with age in our study population. This is in agreement with the poisson regression results for BK (table 2; prevalence ratio (PR) 1.02 per year of age, 95% CI 1.01–1.03), and for the pooled results from all sites, where age is significantly associated with rising RVFV IgG prevalences, both in uni- and in multi-variable regression models (table 3; PR 1.02 per year of age, 95% CI 1.01–1.03). Increasing socio-economic status is associated with decreasing RVFV IgG prevalences (BK site: PR 0.60 per unit, 95% CI 0.40–0.90), whereas gender appears not to influence RVFV IgG prevalence in the study population (uni-variable PR for male gender as compared to female in BK, 1.0, 95% CI 0.61–1.65).

According to the multi-variable models, cattle ownership is significantly associated with RVFV seroprevalence, both in BK and in all sites (PR 1.81, 95% CI 1.15–2.85 for BK; PR 1.76, 95% CI 1.15–2.71 for all sites), although it's uni-variable association in BK is far from significant. Cattle density per square kilometer is a significant prognostic factor in all sites (PR 2.06 per 100/skm, 95% CI 1.64 to 2.59, multi-variable model), including BK, where mean cattle density is higher than in the other sites.

Due to collinearity between the examined environmental variables, these could not be simultaneously included into one model, but were entered one at a time into multi-variable models that were adjusted for age, SES, cattle ownership, and – for the all-sites pooled model – cattle density. Of these environmental variables, vegetation density (EVI) results in the model with the best fit, both in BK and in the pooled analysis. However, most other environmental factors are also strongly associated with RVFV IgG prevalence. It is noteworthy though, that maximum and average land surface temperature (LST) during the day have significant negative associations (PR 0.87 per °C, 95% CI 0.81–

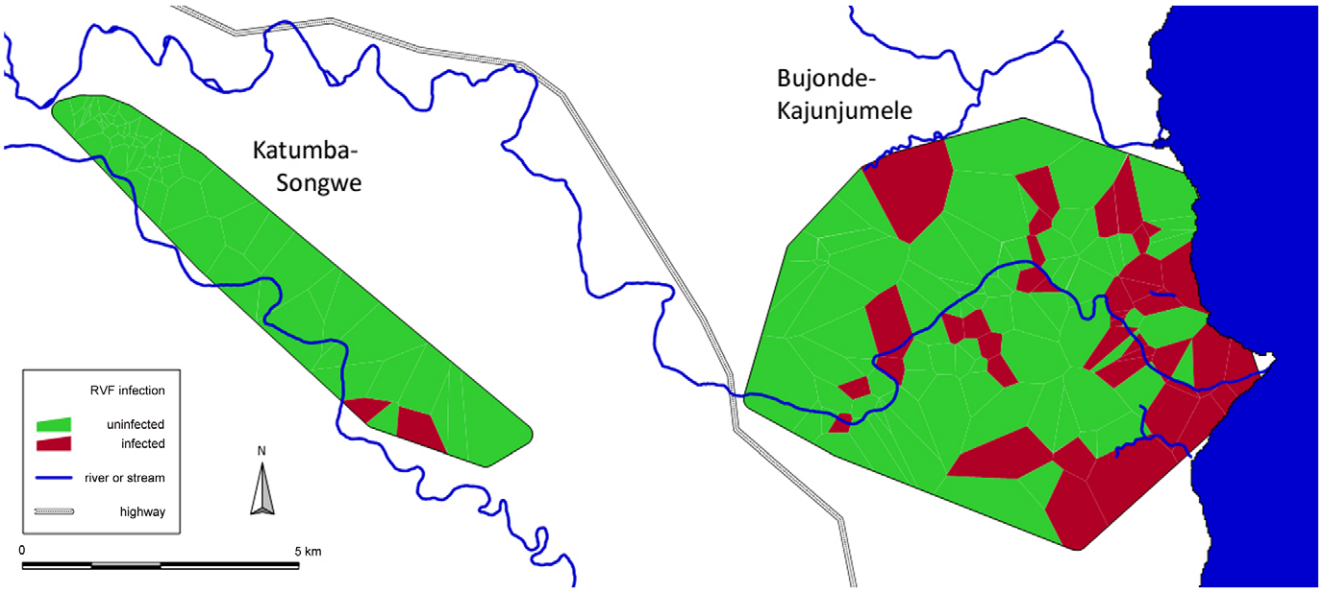

Figure 3. Location of households with RVF IgG-positive participants in Katumba-Songwe and Bujonde-Kajunjumele (BK) sites. Location of households with RVF IgG-positive participants displayed as Voronoi polygons.
doi:10.1371/journal.pntd.0001557.g003

0.94 for max. LST; PR 0.73, 95% CI 0.61–0.86 for average day LST, both multi-variable for BK), whereas average LST during the night has a positive association with RVFV seroprevalence (PR 2.51 per °C, 95% CI 0.94–6.70). Minimum LST was less strongly associated than the other LST variables in the pooled analysis and unrelated to RVFV IgG prevalence in BK site.

Other factors that we examined (population density and the ownership of livestock other than cattle) do not show any strong associations with RVFV infection in our study population (data not shown).

Association with other mosquito-borne and water-borne infections

Within the EMINI study population our group also collected data on chikungunya virus IgG, *P. falciparum* malaria (ICT Malaria P.f./P.v. ICT Diagnostics, Cape Town, South Africa) and presence of *W. bancrofti* filarial antigen (TropBio® Og4C3 serum ELISA, Townsville, Australia). We found that on a household level, RVFV IgG positivity was strongly associated with chikungunya virus IgG in BK site and in all other sites (PR = 4.3; 95% CI 2.3–8.1; PR = 5.3, 95% CI 2.1–13.5, respectively), and with filarial antigen (PR = 2.2; 95% CI 1.3–3.7) and P.falciparum malaria (PR = 4.2, 95% CI 3.3–5.5) in BK. No association was found with *Schistosoma haematobium* infection in BK, nor in the other sites.

Figure 4. Surface Water Collections in Bujonde-Kajunjumele Site. Surface water collections situated close to Lake Malawi. At the end of the rainy season in April, the high prevalence area close to Lake Malawi, with elevations barely above the Kiwira river water level, is characterized by abundant waterlogging, with surface water between homesteads.
doi:10.1371/journal.pntd.0001557.g004

Discussion

The presented analyses identify several socio-demographic and environmental factors that are strongly associated with RVFV seropositivity in our study population. As prevalences in all sites apart from BK are relatively low, we were unsure whether the few cases in the non-BK sites were autochthonous or imported cases and whether an overall analysis of all sites would really yield credible results. It is therefore reassuring that the results of the BK only analysis and those for all sites are similar.

Socio-economic variables

Figure 4 shows that RVFV seroprevalence increases with age, which is in line with the regression results for BK and for all sites. This suggests an endemic circulation of RVFV in our study area, rather than a single outbreak event as reason for the detected seroprevalence.

The inverse association of SES with RVFV IgG means that more affluent people are at lower risk of infection. This has been described for many different infectious diseases in a wide range of settings. Importantly, cattle ownership was not used for SES calculation, as it is a direct risk factor.

Environmental variables

Despite the strong associations that we found for age and SES, most of the examined environmental variables were still significantly associated with RVFV IgG prevalence, when adjusted for possible socio-economic confounding in the multi-

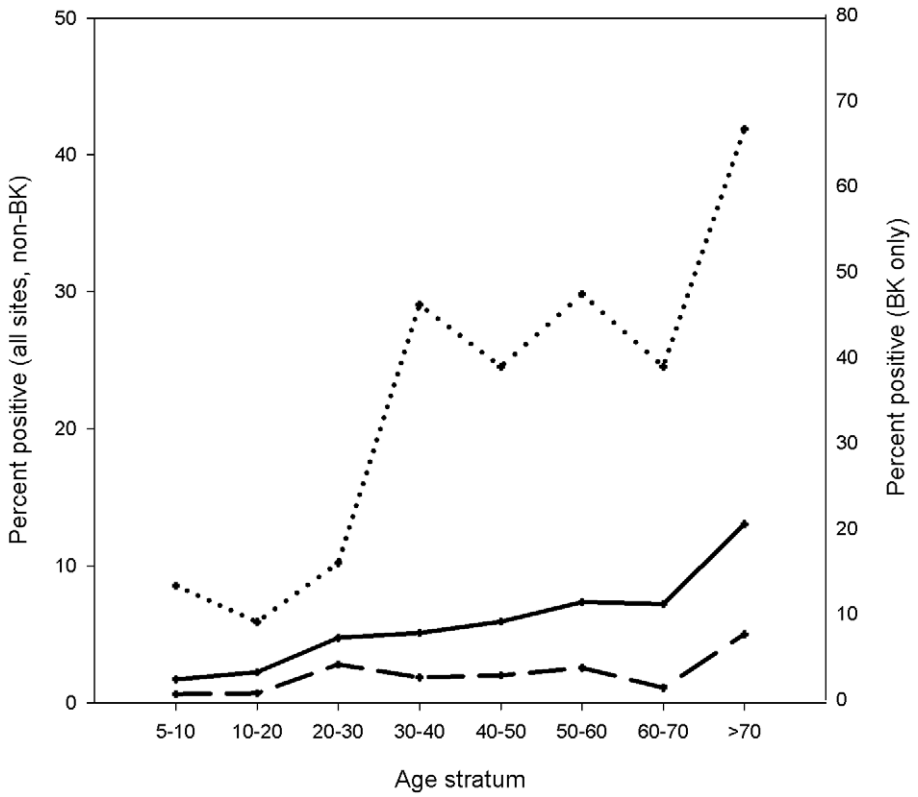

Figure 5. Seropositivity of RVFV IgG by Age. Dotted line: percent positives in BK site, N = 150. Solid line: percent positives in all sites including BK, N = 1228. Dashed line: percent positives in all sites other than BK, N = 1078. Please note the different scales for BK (right axis) and for all sites and non BK sites (left axis).
doi:10.1371/journal.pntd.0001557.g005

variable models showing that their association with RVF is independent.

Our findings that cattle ownership and density of cattle in the area are important factors for RVFV seropositivity were to be expected, since ruminants are the main animal host of RVFV [4]. Cattle owners in BK have the habit of tethering their animals on the doorsteps of their houses at night for fear of theft, providing an animal reservoir of RVFV in proximity of humans, and reportedly increasing the number of *Culex* mosquitoes in the house [24].

Some previously described risk factors for RVF were confirmed in our study: dense vegetation and proximity to perennial water bodies were found associated with RVFV seropositivity in ruminant herds in the Senegal and in humans in Gabon [13,25]. These and other risk factors seem to make the BK site uniquely favorable for human and animal RVFV infection.

In BK, only the low-lying areas close to Lake Malawi are subjected to regular flooding during the rainy season, whereas areas further away from the lake and at slightly higher elevation are not flooded. This phenomenon provides abundant mosquito breeding places in low-lying areas, and is a likely reason for the strong negative association of altitude with RVFV seropositivity, that – in BK site – is already visible on a per meter scale, and for the association with distance from the lake. Frequent waterlogging has lead to large areas of BK site being used for wetland rice cultivation. One report from the 2006–2007 outbreak in Kenya found that soils that retain water were more frequently found in RVF-affected areas than in other areas [26].

Over the entire study area with an altitude range of 479 to 2313 m, it seems obvious that higher elevation negatively affects mosquito breeding and survival.

The association of RVFV seropositivity with other mosquito-borne diseases transmitted by *Anopheles*, *Aedes* and *Culex* species is in agreement with above considerations regarding the role of mosquito favorable habitats as an important factor contributing to RVF prevalence in BK site. However, in our study, RVFV seropositivity is not associated with *S. haematobium*, a water-borne disease. *Bulinus* snails, the intermediate hosts of *S. haematobium*, require permanent water-bodies [27]. Thus, RVFV infection in BK does not seem to depend on proximity to Lake Malawi itself, which is a reservoir for *S. haematobium*, but is more likely a consequence of the seasonal surface water collections that are more common close to the lake. Although it is difficult to single out the predominant factors causing the observed difference between BK and the neighboring low-prevalence site Katumba-Songwe, we presume that the difference in altitude and distance to the lake, and their impact on surface water collections, are the most important reasons.

According to our results, RVFV seropositivity seems to be associated with an optimum temperature range. An adverse effect of low temperatures has been shown for RVFV replication and infectiousness, e.g. in the vector *Culex pipiens* [28,29,30], while higher temperatures above 27–32°C adversely affect hatching success and size of adult *Aedes aegypti* mosquitoes [31]. The correlation with EVI may be explained by dense vegetation protecting water pools from being heated in the sunlight, and from cooling off at night. Furthermore, vegetation density can be regarded as a proxy for the presence of water. Seasonal increases in vegetation are associated with RVF outbreaks on a larger scale and are used for predicition [32,33]. Our results confirm this association on a small scale.

Table 2. Socio-economic and environmental factors and association with RVFV IgG[a] in BK.

Covariate		univariable			multivariable[b d]		
	stratum	PR	(95%CI)	p	PR	(95%CI)	p
Gender[b]							
	female[c]	1					
	male	1.00	(0.61 to 1.65)	0.985			
Age							
	per year	1.02	(1.01 to 1.04)	<0.001	1.02	(1.01 to 1.03)	<0.001
SES score							
	per unit	0.53	(0.34 to 0.84)	0.006	0.60	(0.40 to 0.90)	0.014
Cattle owned							
	no[c]	1			1		
	yes	1.23	(0.74 to 2.05)	0.421	1.81	(1.15 to 2.85)	0.010
Cattle per sqkm[b]							
	per 100	0.84	(0.48 to 1.45)	0.522			
Vegetation (EVI)							
	per unit	3.98	(1.84 to 8.61)	<0.001	2.99	(1.34 to 6.65)	0.007
					Results for other environmental variables when included into the above model instead of vegetation:		
Dist. to Lake Malawi[d]							
	per km	0.74	(0.64 to 0.86)	<0.001	0.79	(0.69 to 0.90)	<0.001
Elevation[d]							
	per m	0.84	(0.77 to 0.93)	<0.001	0.87	(0.80 to 0.94)	0.001
LST maximum[d]							
	per °C	0.84	(0.77 to 0.92)	<0.001	0.87	(0.81 to 0.94)	<0.001
LST average day[d]							
	per °C	0.67	(0.56 to 0.80)	<0.001	0.73	(0.61 to 0.86)	<0.001
LST average night[d]							
	per °C	3.82	(1.28 to 11.45)	0.017	2.51	(0.94 to 6.70)	0.066
LST minimum[d]							
	per °C	1.18	(0.94 to 1.49)	0.149	1.07	(0.88 to 1.31)	0.473

N for BK site = 150.
PR = prevalence ratio; SES = socio-economic status; skm = square kilometre; EVI = enhanced vegetation index; LST = land surface temperature.
[a]results of uni- and multivariable poisson regression with robust variance estimates adjusted for clustering within household.
[b]gender and cattle per skm were not included into multivariable model due to lack of significance.
[c]reference stratum.
[d]to avoid collinearity problems, the environmental variables (vegetation, distance to lake, elevation and the four LST variables) were entered separately into models adjusted for age, SES, and cattle ownership. Multivariable results for these three adjustment variables are those for the model that included vegetation.
doi:10.1371/journal.pntd.0001557.t002

It is a limitation of this study that only serologic findings were available for analysis, specific questioning regarding RVF-related symptoms and sequelae to assess clinical significance of serological findings was not possible because samples were analysed retrospectively. However, conduct of this study within the very well characterised EMINI cohort allowed for a detailed analysis of socio-economic, spatial and ecological covariates on a small scale. Given the persistence of IgG responses over several years, the actual date of infection cannot be deduced from these examinations, and socio-economic and environmental conditions at the time of infections may have differed from the time of participant assessment for EMINI. The presence of RVFV IgG in the younger age groups suggests an ongoing or recent virus circulation.

Public health significance

There are no previous reports of RVF in Mbeya region, and to our knowledge the disease was never diagnosed clinically in Kyela. Since no virus isolation has yet been done in our study, it remains to be elucidated whether the cycling virus is a less virulent RVFV strain such as the apathogenic "clone 13" from the Central African Republic [34,35], or whether acute cases of RVF have been overlooked in the past. Taking into account the relatively high prevalence for malaria and HIV in the area [36], RVFV encephalitis, retinitis and hemorrhagic fever would be comparatively rare events, which may have been misdiagnosed as malaria- or HIV related morbidity, as is often the case with febrile illnesses in malaria-endemic areas [37,38].

Conclusion

In conclusion, this study finds a relatively high RVFV IgG prevalence in an area without previous reports of RVF, and identifies several environmental factors that are associated with RVF infection, independently of age and socio-economic status.

Table 3. Socio-economic and environmental factors and association with RVFV IgG[a] in all sites, including BK.

Covariate		univariable			multivariable[b]		
	stratum	PR	(95%CI)	p	PR	(95%CI)	p
Gender[b]							
	Female[c]	1					
	male	1.12	(0.69 to 1.82)	0.643			
Age							
	per year	1.02	(1.01 to 1.04)	<0.001	1.02	(1.01 to 1.03)	<0.001
SES score							
	per unit	0.31	(0.20 to 0.48)	<0.001	0.51	(0.33 to 0.78)	0.002
Cattle owned							
	no[c]	1			1		
	yes	1.73	(1.05 to 2.86)	0.033	1.76	(1.15 to 2.71)	0.010
Cattle per sqkm							
	per 100	1.67	(1.39 to 2.00)	<0.001	2.06	(1.64 to 2.59)	<0.001
Vegetation (EVI)							
	per unit	6.31	(3.68 to 10.81)	<0.001	2.94	(1.87 to 4.63)	<0.001
					Results for other environmental variables when included into the above model instead of vegetation:		
Elevation[d]							
	per 100 m	0.79	(0.74 to 0.84)	<0.001	0.85	(0.79 to 0.90)	<0.001
LST maximum[d]							
	per °C	0.88	(0.83 to 0.92)	<0.001	0.87	(0.83 to 0.92)	<0.001
LST average day[d]							
	per °C	0.88	(0.82 to 0.94)	<0.001	0.83	(0.76 to 0.92)	<0.001
LST average night[d]							
	per °C	1.46	(1.32 to 1.63)	<0.001	1.31	(1.81 to 1.46)	<0.001
LST minimum[d]							
	per °C	1.45	(1.18 to 1.79)	<0.001	1.21	(0.99 to 1.47)	0.063

N for all sites = 1228.
PR = prevalence ratio; SES = socio-economic status; skm = square kilometre; EVI = enhanced vegetation index; LST = land surface temperature.
[a]results of uni- and multivariable poisson regression with robust varicance estimates adjusted for clustering within household.
[b]gender was not included into multivariable model due to lack of significance not included into multivariable model due to lack of significance.
[c]reference stratum.
[d]to avoid collinearity problems, the environmental variables (vegetation, elevation and the four LST variables) were entered separately into models adjusted for age, SES, cattle ownership and cattle density. Multivariable results for these four adjustment variables are those for the model that included vegetation.
doi:10.1371/journal.pntd.0001557.t003

If confirmed in future studies, these findings have important implications in the areas close to Lake Malawi, where health facilities and their staff should be made aware of RVF as a possible diagnosis for their patients. The environmental risk-factors for RVF infection that we identified could serve to predict areas of RVFV endemicity, in addition to outbreak prediction which can be done based on rainfall and vegetation data. It would be interesting to do further studies in similar high risk areas, since it is likely that undetected endemic cycling of RVFV is occurring in many areas apart from our study site.

Supporting Information

Checklist S1 STROBE checklist.
(DOC)

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Author Contributions

Conceived and designed the experiments: NH ES PC TL GD MH. Performed the experiments: NW PC IK GD. Analyzed the data: NH ES NW GD MH. Contributed reagents/materials/analysis tools: ES PC EN HM LM. Wrote the paper: NH ES GD PC. Acquisition of funding: MH NH. Supervision of acquisition of data: NH ES PC EN HM LM GD.

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The molecular bacterial load assay replaces solid culture for measuring early bactericidal response to antituberculosis treatment.

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The Molecular Bacterial Load Assay Replaces Solid Culture for Measuring Early Bactericidal Response to Antituberculosis Treatment

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We evaluated the use of the molecular bacterial load (MBL) assay, for measuring viable *Mycobacterium tuberculosis* in sputum, in comparison with solid agar and liquid culture. The MBL assay provides early information on the rate of decline in bacterial load and has technical advantages over culture in either form.

Assessment of *Mycobacterium tuberculosis* sputum bacterial load has routinely been performed using solid culture (1). Automated liquid culture has been proposed as an alternative (2), but all culture-based methods are hampered by contamination with other microorganisms (3), protracted time to obtain results (4), and populations of viable, nonculturable bacteria (5, 6). We recently described the molecular bacterial load (MBL) assay, based on 16S rRNA, observing that MBL declined biphasically in response to treatment (7). In the present study, we directly compared the MBL assay with solid and liquid culture on 148 sputum samples (collected overnight from 1600 h until 0800 h the following morning) from 20 patients enrolled and hospitalized at the EBA Unit, Tanzanian National Institute for Medical Research-Mbeya Medical Research Centre, Tanzania. Samples were collected pretreatment (2 overnight samples) and longitudinally (days 2, 4, 5, 7, 10, and 14) during standard WHO treatment for drug-sensitive tuberculosis. The OEBA (Observation of Early Bactericidal Activity) study was a capacity building project of the PanACEA consortium. Before patient enrolment, the study was approved by the site's local ethics board (reference MRH/T.30/44/2) and national ethics board (reference NIMR/HQ/R.8a/Vol.IX/1169) and the sponsor (University of Munich) ethics board. The study was conducted in compliance with the declaration of Helsinki. All patients provided written informed consent to study participation, including use of their samples for evaluation of novel molecular assays. The study was registered in the Pan African Clinical Trials Registry (pactr.org) under PACTR201209000394102.

Sputum was homogenized, and one half was decontaminated with *N*-acetyl-L-cysteine-sodium hydroxide (1% final NaOH concentration) prior to inoculation into mycobacterial growth indicator tubes (BBL MGIT; Becton, Dickinson and Company, MD, USA), according to the manufacturer's instructions, in order to determine time to positivity (TTP) in the Bactec MGIT 960 (Becton, Dickinson and Company, MD, USA). The second portion was mixed with an equal volume of Sputasol (Oxoid, Limited, United Kingdom), and quadruplicate 100-μl volumes of 10-fold dilutions were inoculated onto 7H11 agar containing Mycobacteria Selectatab Kirchner supplement (Mast Group, Limited, United Kingdom). Nine hundred fifty microliters of sputum/Sputasol was preserved in guanidine thiocyanate containing 1% β-mercaptoethanol, and the MBL assay was performed as detailed in the work of Honeyborne et al. (7). MBL value was assigned based on 16S rRNA cycle threshold (C_T) using the normalized 16S rRNA C_T in the following equation: bacterial load ($\log_{10}$) = (normalized 16S $C_T - 31.76$)/-3.171 (7).

Artificial sputum (see Text S1 in the supplemental material) developed as a matrix for high assay standards was found to be a good substitute for pooled human sputum. High standards had mean 7.46 $\log_{10}$ bacilli (standard error of the mean [SEM], 0.08) and 7.49 $\log_{10}$ bacilli (SEM, 0.07) ($P = 0.80$) for pooled human sputum and artificial sputum, respectively (see Fig. S1 in the supplemental material). The low standard mean of 3.70 $\log_{10}$ bacilli (SEM, 0.13) for pooled human sputum was statistically different from that of artificial sputum (mean, 3.38 $\log_{10}$ bacilli; SEM, 0.13; $P = 0.01$) (see Fig. S1). However, the SEMs are identical for the two matrices, and therefore, the critical factor, reproducibility of extraction of an assigned bacterial number, was met.

Spearman rank correlations for 148 sputum samples revealed a high degree of correlation between the assays: $\log_{10}$ MBL compared to solid agar $\log_{10}$ CFU ($r = +0.84$; 95% confidence interval [CI], 0.78, 0.88), $\log_{10}$ MBL compared to solid agar $\log_{10}$ TTP ($r = -0.81$; 95% CI, -0.86, -0.74), and $\log_{10}$ TTP compared to solid agar $\log_{10}$ CFU ($r = -0.78$; 95% CI, -0.84, -0.71; for all, $P < 0.0001$) (see Fig. S2 and Text S2 in the supplemental material). Correlation between $\log_{10}$ CFU and $\log_{10}$ TTP for our study was comparable to that in a study of >2,000 sputum samples tested in South Africa ($r = -0.72$) (2), although TTP was not log-transformed in this study.

Direct comparison of solid culture and the MBL assay for individual patients revealed that the declines in bacterial load closely

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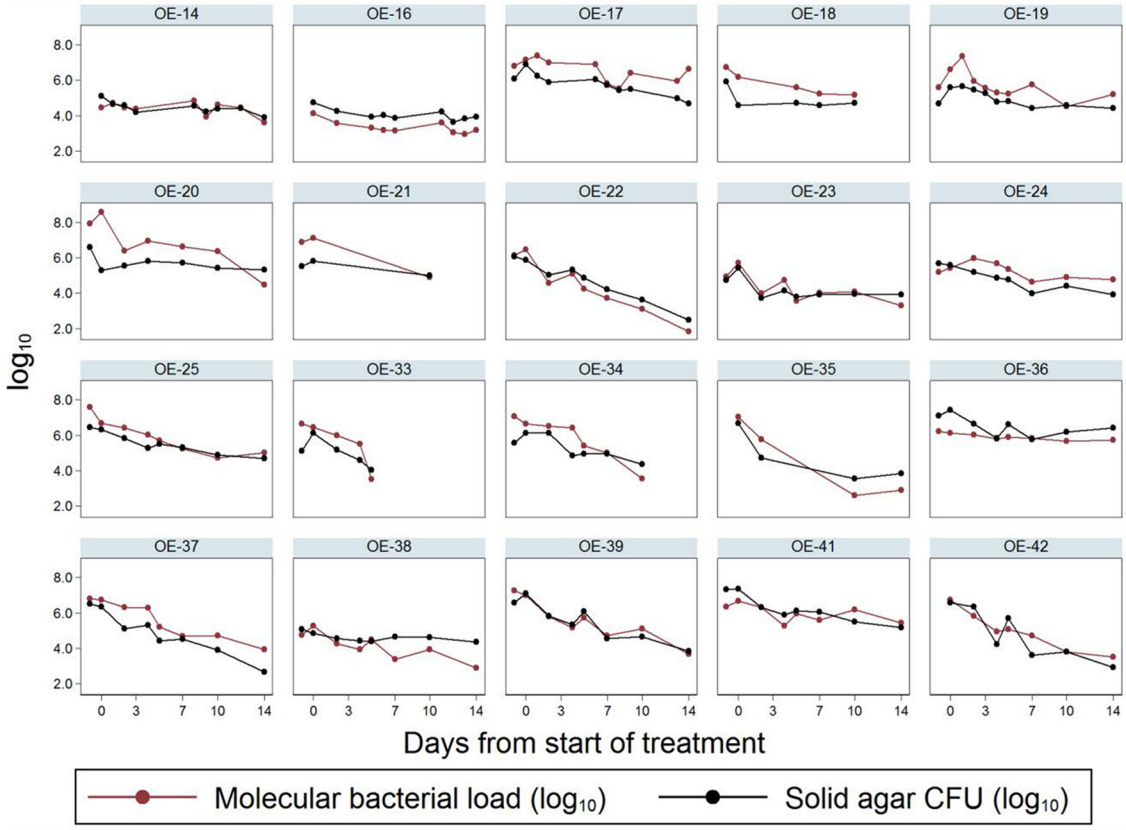
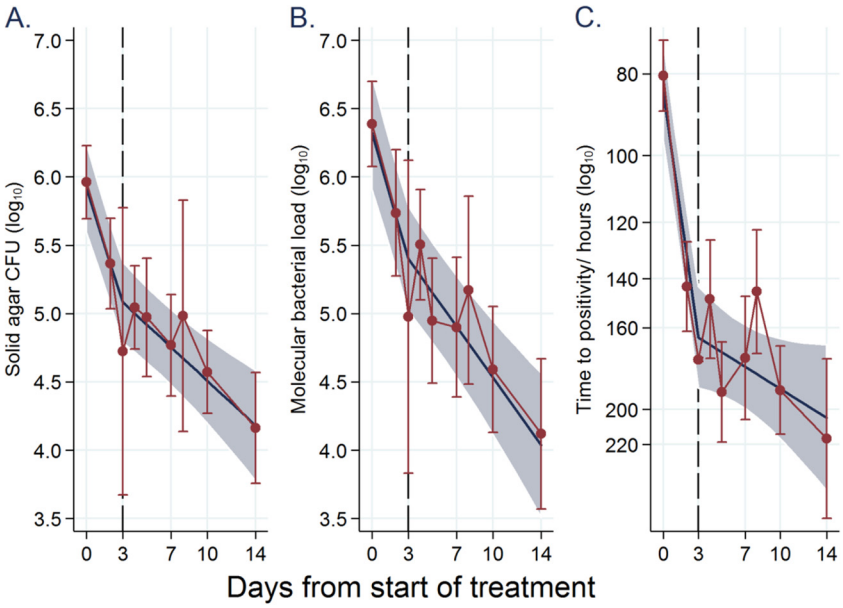


FIG 1 Individual patient plots comparing bacterial load measured using solid agar and MBL during the first 14 days of treatment for 20 individuals.

matched during the first 14 days of therapy (Fig. 1). rRNA is downregulated in bacteria that are entering dormancy (8), and so one might expect that the MBL assay is an underestimate of bacterial load. However, the baseline median bacterial load was 0.43 $\log_{10}$ (95% CI, 0.12, 0.73; $P = 0.008$) higher when measured using the MBL assay than when measured by the solid agar CFU method (paired t test, $P = 0.008$). This may be explained by the selective antibiotics in the solid agar method killing a proportion of the bacteria present. We observed a similar effect for six clinical isolates (*M. tuberculosis* [$n = 5$] and *Mycobacterium bovis* [$n = 1$]) isolated from decontaminated sputum on Lowenstein-Jensen (LJ) slopes (Southern Group Laboratories, Limited, United Kingdom). Determination of bacterial number for these was done using the Miles-Misra method (9) on 7H10 agar with 10% oleic acid-albumin-dextrose-catalase (OADC) supplement (Becton, Dickinson and Company, MD, USA) with and without the addition of antibiotics using bacteria subcultured from the LJ slope into 7H9 medium containing 0.2% Tween 80 and 10% ADC supplement (Becton, Dickinson and Company, MD, USA). Addition of antibiotics (Mycobacteria Selectatabs Kirchner; Mast Group, Limited, United Kingdom) reduced the bacterial count by 0.47 $\log_{10}$ (95% CI, 0.23, 0.71; paired t test, $P = 0.004$). This may explain the increased MBL readout observed here for patient sputum in comparison to that for solid culture despite comparable rates of decline.

A biphasic decline in bacterial load was observed for liquid and solid culture and the MBL assay with a node at day 3 (Fig. 2). Full details of statistical analysis are given in Text S2 in the supplemental material. The decline rate for days 0 to 3 for solid agar was -0.278 $\log_{10}$ day^{-1} (95% confidence interval, -0.345, -0.212), and that for MBL was -0.307 $\log_{10}$ day^{-1} (-0.389, -0.226). MBL decline was therefore slightly quicker over the first 3 days, although the confidence intervals overlap. The use of mRNA for the isocitrate lyase gene and the use of noncoding ribosomal promoter region mRNA as amplification targets for bacterial quantification have previously been found to respond comparably to culture during days 2 to 7 of therapy but not during the first 2 days of therapy (10). In contrast, MBL robustly matched solid and liquid culture over the early phase of treatment. Extended early bactericidal activities (EBAs) from days 3 to 14 were also comparable, with -0.082 $\log_{10}$ (-0.119, -0.045) and -0.124 $\log_{10}$ (-0.170, -0.079) for CFU on solid medium and MBL, respectively. These data match other EBA studies measuring treatment response with solid culture (11–16). The close comparability between MBL and culture contrasts with data for treatment monitoring using the GeneXpert MTB/RIF assay (Xpert; Cepheid, CA, USA). GeneXpert MTB/RIF did not respond to changes in bacterial load as rapidly as did culture (17). Within-subject variation of Xpert was high, at 56.7%. In contrast, MBL had a 9.6% variance between 2 baseline samples for 16 subjects (18), suggesting that MBL is more reproducible. To corroborate this, within-patient variabilities for solid (17.9%) and liquid (21.6%) culture in our study were compared to those in the study by Kayigire et al., with 16.5% and 22%, respectively (18). Prior to exclusion of outliers, solid culture had invalid results in 11% of samples (18 of 169), whereas MBL had only 1.2% of samples (2 of 169) unreadable.



D.

Method	Baseline Mean (std error)	Time period	Slope /day	95% confidence interval, p-value
Solid culture log_{10} cfu mL^{-1} sputum	5.95 (4.73-7.35)	0-3 days	-0.278	(-0.345, -0.212), p<0.0001
		3-14 days	-0.082	(-0.119, -0.045), p<0.0001
		0-14 days	-0.124	(-0.157, -0.091), p<0.0001
MBL log_{10} bacilli mL^{-1} sputum	6.44 (4.11-8.38)	0-3 days	-0.307	(-0.389, -0.226), p<0.0001
		3-14 days	-0.124	(-0.170, -0.079), p<0.0001
		0-14 days	-0.163	(-0.204, -0.122), p<0.0001
TTP liquid culture (hours)	77.5 (59.5-195.0)	0-3 days	0.097	(0.078, 0.116), p<0.0001
		3-14 days	0.009	(-0.0010, 0.0183), p=0.039
		0-14 days	0.028	(0.020, 0.035), p<0.0001

FIG 2 Bacterial load during the first 14 days of treatment: linear mixed-effects modeling using splines (splines allow for estimating different slopes before and after the specified node). (A) CFU on solid culture. (B) MBL. (C) Liquid culture (the scale on the vertical axis for panel C is reversed to aid comparison with panels A and B). (D) Declines of bacteria per day for the 3 methods. The red line shows the raw mean with 95% confidence interval, and the thicker blue line shows the fitted biphasic slope with 95% confidence region.

In summary, our data show that the MBL assay is at least as good as culture for measuring EBA during standard tuberculosis therapy to day 14, with higher precision, fewer missing data, and a shorter time to result (24 h compared to weeks). This assay shows promise as a replacement for culture in future EBA trials testing new drugs.

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A continuously monitored colorimetric method for detection of *Mycobacterium tuberculosis* complex in sputum

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SUMMARY

SETTING: Mbeya, Tanzania.

OBJECTIVE: To develop a new liquid culture method to detect *Mycobacterium tuberculosis* complex (MTC) in sputum using 2,3-diphenyl-5-thienyl-(2)-tetrazolium (STC), the nitrate reductase assay (NRA) and *p*-nitrobenzoic acid (PNB).

DESIGN: Ninety-three sputum samples collected from 18 tuberculosis patients were decontaminated with *N*-acetyl-L-cysteine-sodium hydroxide using MGIT™ 960 and in STC-NRA cultures, both in the presence and in the absence of PNB, an inhibitor of MTC growth. The reduction of STC by colour change indicated mycobacterial growth; NRA was then performed to confirm MTC.

RESULTS: STC-NRA culture was positive for acid-fast bacilli in 66/93 (71%) samples, of which 60/93 (64.5%) were identified as MTC-positive and 6/93 (6.5%) as indeterminate mycobacteria. MGIT indicated MTC in 59/93 (63.4%) cultures. Contamination was detected in 12/93 (13%) STC-NRA cultures vs. 29/93 (31.2%) MGIT cultures. The mean time to detection (TTD) of MTC using STC-NRA was 14 days and 7 days using MGIT.

CONCLUSION: The STC-NRA method is sensitive for the detection of MTC in sputum. TTD increased with duration of anti-tuberculosis treatment, highlighting the value of this method in monitoring treatment success. The method is simple and inexpensive and, unlike MGIT, does not require technical equipment. The preliminary performance characteristics of the method should be further evaluated in larger studies.

KEY WORDS: nitrate reductase assay; liquid culture; 2,3-diphenyl-5-thienyl-(2)-tetrazolium STC; treatment monitoring; TTD

TUBERCULOSIS (TB) is still a major global health problem, particularly in low-resource countries with high human immunodeficiency virus prevalence. In 2011, TB incidence was estimated at 8.7 million cases globally, with the majority occurring in resource-limited settings.[1] To improve TB control, rapid and highly accurate diagnostic tests are needed for the detection of active TB in patients; these tests should be accessible to resource-limited populations.[2]

The definitive diagnosis of TB is established by the isolation and identification of *Mycobacterium tuberculosis* complex (MTC).[3–5] As the use of solid culture media and biochemical and/or molecular identification tests are time consuming and require sophisticated equipment, new approaches based on liquid media and rapid identification tests have been developed for quicker detection of the pathogen. The BD MGIT™ (Mycobacteria Growth Indicator Tube) 960 system (BD, Sparks, MD, USA) is used as a gold standard for MTC detection and testing of susceptibility to anti-tuberculosis antibiotics.[6] Other methods optimised for drug susceptibility testing with limited or no application for the direct detection of MTC include the microscopic-observation drug susceptibility assay (MODS),[7] the nitrate reductase assay (NRA)[8] and colorimetric methods.[9–13] Species confirmation tests include MPT64 antigen-based tests such as the BD MGIT TBc identification test (TBc ID; BD)[14,15] and the use of *p*-nitrobenzoic acid (PNB), which selectively inhibits the growth of MTC but not that of non-tuberculous mycobacteria (NTM).[16–20]

Although the new methods have shown good potential to replace conventional culture on Löwenstein-Jensen medium and classical identification tests, some drawbacks limit their application in low-resource countries. MGIT 960 is costly, and its implementation in low-resource settings with irregular power supplies and limited access to maintenance and required

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consumables might affect its performance.[21,22] Moreover, it requires an additional method for species identification to avoid false-positive diagnoses of TB due to contamination with NTM. MODS is based on the detection of *M. tuberculosis* in liquid culture media using an inverted microscope[7] that is not routinely available in TB laboratories.[23] Colorimetric methods, based on the use of oxidation-reduction (redox) indicators, such as Alamar blue,[9] MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide),[10] XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2h-tetrazolium-5-carboxanilide)[11] and resazurin,[12] and NRA have been optimised to detect *M. tuberculosis* from either isolates or acid-fast bacilli (AFB) smear-positive sputum samples. These indicators and the Griess reagent (used in NRA to detect nitrite) are only added to the liquid culture medium after incubation for 7–28 days.[8,24–26] This time window for the detection and identification of *M. tuberculosis* solely by the reduction of nitrate may yield false-negative results with paucibacillary sputum samples and false-positive results in NTM that reduce nitrate, such as those of the *M. fortuitum* complex.[4,27] Unlike the reagents mentioned above, 2,3-diphenyl-5-thienyl-(2)-tetrazoliumchloride (STC), a redox indicator that changes from colourless to red with the growth of organisms, can be added to the media before inoculating the sample and is therefore more suitable for the continuous monitoring of bacterial growth.[13,28,29] TBc ID is a rapid MPT64-based immunochromatographic test used to confirm the presence of MTC from AFB-positive MGIT culture; this may, however, have limited use due to high costs.

In this study, we combined the use of STC, NRA and PNB to develop the STC-NRA method, a new colorimetric liquid culture method, for continuous monitoring of bacterial growth. This method enables NRA to be performed when micro-organisms have multiplied sufficiently (indicated by the STC colour change). NRA is conducted in both the presence and the absence of PNB. The combined results help in confirming the presence of MTC species.

As time to detection (TTD) of mycobacterial growth in MGIT culture can be used to monitor the bacterial load in TB patients under treatment,[30,31] the STC-NRA TTD method was evaluated in the same group of patients to monitor MTC growth. MGIT 960, with the TBc ID test for species confirmation, was used as the gold standard for the detection and identification of MTC.

MATERIAL AND METHODS

Sputum sample collection

Sputum samples were obtained from TB patients before and during standard anti-tuberculosis treatment per Tanzanian guidelines; the patients were participants in the PanACEA Consortium study entitled 'Observation of early bactericidal activity of standard tuberculosis treatment' (OEBA TB, pactr.org 201209 000394102), at the TB department of the Mbeya Medical Research Center, Mbeya, Tanzania, from 1 July to 1 December 2012. All patients provided consent to participate in the study, including assessment of sputum samples with novel methods.

Two overnight sputum samples were collected before the start of treatment, and 14 overnight sputum samples during the first 2 weeks of treatment. Thereafter, early morning and spot sputum samples were collected weekly until week 8 of treatment. For the present analysis, 93 unselected samples collected from 18 patients before (TB suspects) and during treatment were cultured simultaneously using the MGIT and the STC-NRA methods.

Culture and Mycobacterium tuberculosis *complex identification methods*

Sputum sample processing

Samples were decontaminated using the standard *N*-acetyl-L-cysteine method with sodium citrate and sodium hydroxide (NaOH).[4] The sediment obtained after decontamination was re-suspended in 1.5 ml phosphate buffered saline; 0.1 ml of the decontaminated, concentrated sample was used to prepare a smear for Ziehl-Neelsen staining.

MGIT culture (gold standard)

For MGIT culture, 0.5 ml of the sediment was inoculated into BD MGIT 960 liquid media per the manufacturer's standard culture protocol. Presence of either AFB or contaminant bacteria in MGIT-positive culture was confirmed using smear microscopy and culture on blood agar plates. MGIT-negative cultures (no growth) were reported at 42 days. Species identification of all AFB smear-positive MGIT was performed using the TBc ID test.

STC-NRA culture: preparation of reagents

STC solution: a stock solution of STC was prepared at 5 mg/ml in distilled water, filter-sterilised, aliquoted and kept at −70°C for up to 3 months.

PNB solution: a stock solution of PNB was prepared by dissolving 0.25 g PNB in 1.5 ml 1N NaOH solution and diluted by adding 8.5 ml of sterile distilled water, filter-sterilised, aliquoted and kept at −70°C for up to 3 months.

If storage at −70°C is not available, these reagents can also be prepared daily according to the volume of culture media prepared.

Griess reagent: stock solutions of hydrochloric acid (HCl), sulphanilamide and naphthylethylenediamine dihydrochloride were prepared at respectively 50% volume/volume (v/v), 0.2% weight/volume (w/v) and 0.1% (w/v) in distilled water. The reagents were kept at 2–8°C for 1 month and mixed shortly before use as follows: one part 50% concentrated HCl,

2 parts 0.2% sulphanilamide and 2 parts 0.1% N-1-naphthylethylenediamine dihydrochloride.

Inoculation of decontaminated sputum into STC-NRA media: 0.1 ml of processed samples was inoculated into each of three screwcapped tubes containing STC-NRA, NRA and NRA PNB culture media. The indicator tube containing the STC-NRA culture (Tube C) was prepared as follows: 0.9 ml of Middlebrook 7H9 supplemented with 0.5% (v/v) glycerol, 10% (v/v) oleic acid-albumin-dextrose-catalase (BD), 2% PANTA™ antibiotic mixture (BD), 0.1% (w/v) BD Bacto™ Casitone and 1000 μg/ml of potassium nitrate. STC was added at a final concentration of 50 μg/ml. Tube N (NRA) and Tube N-P (NRA-PNB), used for the parallel detection and identification of MTC, were prepared with the same components as Tube C but without STC; PNB (final concentration 500 μg/ml) was added to Tube N-P. The three tubes were incubated at 37°C and examined every other day to detect any bacterial growth, indicated by a colour change in Tube C. A pink sediment in the indicator Tube C was interpreted as growth; 0.2 ml of freshly prepared Griess reagent was added to tubes N and N-P. The presence of either AFB or contaminant bacteria in Tube N was confirmed using smear microscopy and culture on blood agar plates. The interpretation of STC-NRA and MGIT culture results are shown in Table 1; Figure 1 shows selected results.

Statistical analysis

Data were tabulated and analysed using Microsoft Excel spreadsheets (Microsoft, Redmond, WA, USA) and SPSS version 15.0 (Statistical Product and Service Solutions, Chicago, IL, USA). The mean TTD (m-TTD) between both cultures was compared using the Wilcoxon test; 33 samples with contaminated MGIT or STC-NRA culture were excluded from the m-TTD analysis. Correlation between treatment days and the TTD of MTC and mycobacteria for the STC-NRA and MGIT cultures was calculated using the non-parametric Spearman's rank correlation coefficient (ρ); statistical significance was assumed at $P \leqslant 0.05$.

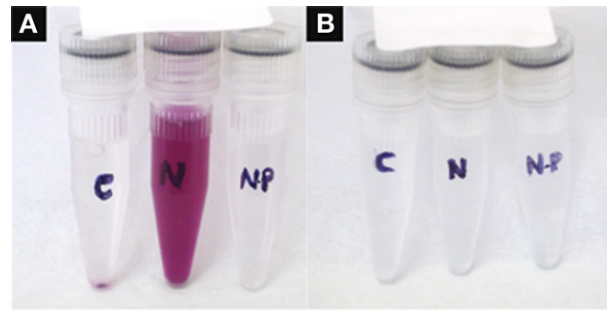

Figure 1 Interpretation of the STC-NRA culture results. **A**. Positive for *M. tuberculosis* complex, growth indicated by colour change (pink) in Tube C (small sediment at the bottom), positive NRA in Tube N, confirmation by positive ZN smear (not shown). **B**. Negative culture for mycobacteria, no growth in Tube C. STC = 2,3-diphenyl-5-thienyl-(2)-tetrazolium; NRA = nitrate reductase assay; ZN = Ziehl-Neelsen. This image can be viewed online in colour at http://www.ingentaconnect.com/content/iuatld/ijtld/2013/00000017/00000012/art00019

RESULTS

Overall, 93 sputum samples collected from 18 patients were decontaminated and concentrated for the preparation of smear and culture simultaneously in MGIT and STC-NRA. The results were classified according to the described algorithm (Table 1). Contamination rates were 12/93 samples (13%) in STC-NRA

Table 1 Interpretation of STC-NRA and MGIT culture results

Culture STC-NRA*			Confirmation tests		
Tube C	Tube N	Tube N-P	Blood agar†	ZN smear†	Final culture results
Colourless	NA	NA	NA	NA	No growth‡
Pink sediment	Pink	Colourless	Negative	AFB-positive	MTC-positive
Pink sediment	Pink	Pink	Negative	AFB-positive	NTM-positive
Pink sediment	Pink	Pink	Positive	AFB-positive/negative	Contaminated
Pink sediment	Colourless	Colourless	Negative	AFB-positive	Indeterminate mycobacteria§
MGIT instrument					
Negative			NA	NA	No growth
Positive			Negative	AFB-positive	MTC-positive¶
Positive			Negative	AFB-positive	NTM-positive¶
Positive			Positive	AFB-positive/negative	Contaminated

*Tube C: pink sediment precipitation when STC is reduced by bacterial growth; Tube N: colour change (pink) when reduction of nitrate by mycobacteria was detected by adding Griess reagent to the media; Tube N-P, no colour change when nitrate is not reduced by MTC in the presence of PNB and colour change (pink) when nitrate is reduced by NTM in the presence of PNB.
†Test performed in Tube N using the STC-NRA method and in the MGIT tube.
‡For all negative Tube C results at 56 days, Griess reagent was added to Tube N and Tube N-P to confirm the presence of mycobacteria.
§Determined after a small amount of zinc powder was added to Tube N to confirm that nitrate had not been reduced by micro-organism, to rule out a false-negative NRA.
¶Mycobacteria isolated from MGIT tube were identified using the TBc ID test.
STC = 2,3-diphenyl-5-thienyl-(2)-tetrazolium; NRA = nitrate reductase assay; MGIT = Mycobacteria Growth Indicator Tube; ZN = Ziehl-Neelsen: NA = not applicable; AFB = acid-fast bacilli; MTC = *M. tuberculosis* complex; NTM = non-tuberculous mycobacteria; PNB = *p*-nitrobenzoic acid.

Table 2 Results of STC-NRA culture vs. MGIT culture using the TBc ID test as gold standard

	STC-NRA culture, *n*					
	MTC-positive*	NTM-positive*	Indeterminate mycobacteria†	No growth	Contaminated	Total
MGIT culture results						
MTC-positive*	47	0	6	2	4	59
NTM-positive*	0	0	0	0	0	0
No growth	0	0	0	5	0	5
Contaminated	13	0	0	8	8	29
Total	60	0	6	15	12	93

*AFB isolated from MGIT culture were identified as MTC or non-tuberculous mycobacteria using the TBc ID test; AFB isolated from STC-NRA culture were identified by the reduction of nitrate with and without PNB.
†Six AFB-positive isolates did not reduce nitrate, and were declared 'indeterminate mycobacteria' following definitions in Table 1. All isolates obtained in MGIT tubes from the same samples were identified as MTC.
STC = 2,3-diphenyl-5-thienyl-(2)-tetrazolium; NRA = nitrate reductase assay; MGIT = Mycobacteria Growth Indicator Tube; MTC = *M. tuberculosis* complex; NTM = non-tuberculous mycobacteria; AFB = acid-fast bacilli; PNB = *p*-nitrobenzoic acid.

and 29/93 (31.2%) in MGIT. MGIT culture was MTC-positive in 59/93 (63.4%) samples; STC-NRA showed AFB growth in 66/93 (71%), of which 60/93 (64.5%) were identified as MTC-positive and 6/93 (6.5%) classified as indeterminate mycobacteria following the results of NRA in tubes N and N-P (Table 2). No MGIT or STC-NRA cultures grew NTM strains.

The results were analysed again using the smear microscopy status of the sputum submitted. Smear-positive samples (n = 73) were MTC-positive in 54 (74%) MGIT cultures and MTC-positive in 56 (76.7%) STC-NRA cultures; smear-negative samples (n = 20) showed MTC growth in 5 (25%) MGIT cultures and in 4 (20%) STC-NRA cultures.

In the 60 cultures compared for this readout, TTD was significantly longer in STC-NRA cultures than in MGIT; m-TTD was 14 days in STC-NRA and 7 days in MGIT ($P < 0.01$; Figure 2). All samples (15/93) with a negative Tube C (i.e., colourless) failed to reduce nitrate in Tube N and Tube N-P. The duration of anti-tuberculosis treatment correlated significantly with m-TTD for MGIT ($\rho = 0.94$), and with m-TTD in STC-NRA ($\rho = 0.87$; $P < 0.01$; Figure 3). After day 3 of treatment, the STC-NRA curve showed greater variability than the MGIT curve; however, due to the low sample number per timepoint of one to five, this should be interpreted with caution.

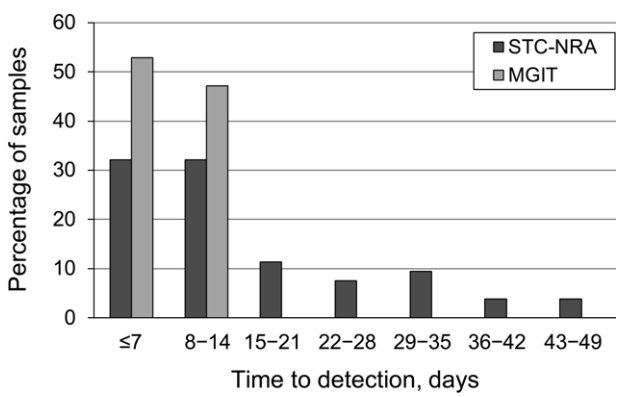

Figure 2 Time to detection of *M. tuberculosis* complex and mycobacteria with STC-NRA and MGIT cultures. STC = 2,3-diphenyl-5-thienyl-(2)-tetrazolium; NRA = nitrate reductase assay; MGIT = Mycobacteria Growth Indicator Tube.

DISCUSSION

The study has shown that the STC-NRA culture method is feasible, and that its sensitivity in detecting MTC in sputum samples seems to be similar to MGIT. The use of STC allowed easy visual monitoring of cultures for growth throughout the entire incubation period of 56 days, as, unlike resazurin, it is reduced exclusively by bacterial growth and not by components of the Middlebrook 7H9 liquid medium (G Rojas-Ponce, unpublished data). The longer TTD in STC-NRA than in MGIT cultures in this study may be due to the smaller inoculum cultured into STC-NRA (one fifth of the volume of the MGIT tubes). It is also possible that STC requires more organisms, and therefore a longer incubation period, to develop a visible colour change than that required by the MGIT system for the photometric detection of fluorescence.

The species identification of MTC using nitrate reduction and PNB, triggered by positivity of the indicator tube, could replace the need to use additional identification tests. In this study, six mycobacterial isolates from different patients were not identified as MTC by NRA and PNB. However, as isolates obtained in MGIT culture from the same samples were identified as MTC and the previous and/or subsequently submitted samples of the patients in question yielded isolates identified as MTC by STC-NRA, we believe that these isolates were in fact MTC, and NRA may have been false-negative due to anti-tuberculosis treatment. In such doubtful cases, an MPT64 antigenic speciation test such as TBc ID should be included in the algorithm in future studies to confirm the presence of MTC from Tube C. If future studies confirm the validity of STC-NRA species identification, it may be

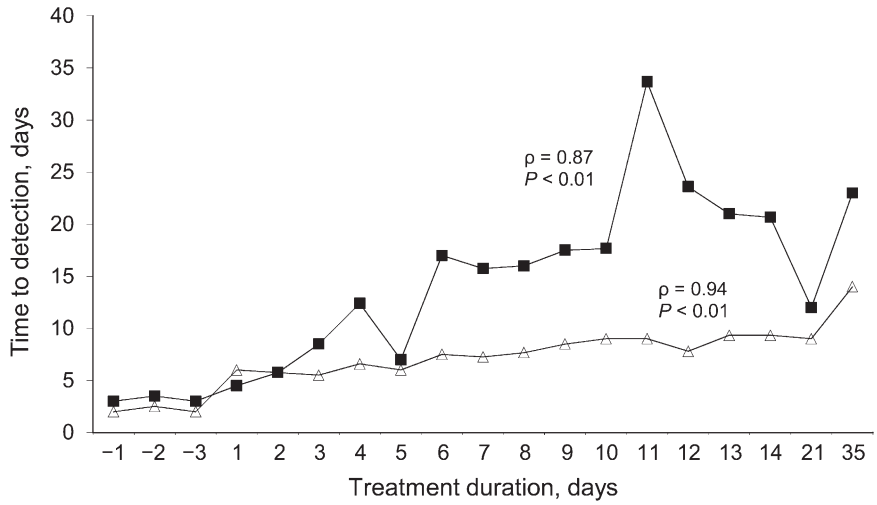

Figure 3 Correlation between TTD and treatment duration. The mean TTD of *M. tuberculosis* complex and mycobacteria in STC-NRA (■) and MGIT (Δ) methods was calculated from the total number of samples at each treatment point (min 1, max 5); coefficient for correlation (ρ). TTD = time to detection; STC = 2,3-diphenyl-5-thienyl-(2)-tetrazolium; NRA = nitrate reductase assay; MGIT = Mycobacteria Growth Indicator Tube.

possible to omit AFB staining from positive cultures with a valid NRA reading.

Our centre, which is located in a subtropical region, has a relatively high contamination rate in liquid culture, similar to other centres in the area. Collection of samples in dusty environments and long distances for transportation to the centre may contribute to this. Previous studies performed in similar settings, such as Zambia,[32] Gambia[33] and Kenya,[20,34] have also reported contamination to be a serious problem when evaluating new diagnostic methods, and have mentioned technical problems, bacteria in the patients' mouths, environmental bacteria and fungi as possible reasons. It is encouraging to observe that the contamination rate was considerably lower in STC-NRA than in MGIT.

Regarding biosafety, liquid culture-based systems require biosafety level 3 laboratory facilities,[21] as the manipulation of liquid cultures can generate more infectious aerosols than solid cultures. The volume of media (1 ml), and thus the mycobacterial load, is smaller in STC-NRA than in MGIT and may result in a lower risk of generating infectious aerosols. However, further studies on the determination of mycobacterial load at the time of positivity are needed to recommend a realistic and affordable biosafety level, mainly in low-resource countries, for this new test.

Our study is limited by the small number of samples collected from each patient at each time point. Likewise, the application of this new test only in a patient population with already confirmed TB under treatment limited the evaluation of its specificity and diagnostic sensitivity. The fact that no NTM grew on culture in our study means that no conclusion can be drawn concerning the specificity of NRA species identification in NTM-positive cultures. Future diagnostic studies should also experiment with the simultaneous detection of drug resistance, e.g., by adding two tubes including rifampicin and isoniazid to the primary culture in analogy to MODS.

In conclusion, this study provides proof of concept for STC-NRA as the first colorimetric method for the continuous monitoring of MTC growth in sputum culture. Our data show that the sensitivity of the method was similar to that of MGIT, and that it has potential for the monitoring of treatment success. Larger studies are required to evaluate its performance as a method for the diagnosis of TB as well as for monitoring treatment success.

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Conflict of interest: none declared.

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RÉSUMÉ

CONTEXTE : Mbeya, Tanzanie.
OBJECTIF : Elaborer une nouvelle culture sur milieu liquide pour détecter le complexe *Mycobacterium tuberculosis* dans les crachats au moyen du 2,3-diphényl-5-thienyl-(2)-tetrazolium (STC), du test de la nitrate réductase (NRA) et de l'acide para-nitrobenzoïque (PNB).
SCHÉMA : On a décontaminé par NALC-NaOH 93 échantillons de crachats provenant de 18 patients TB et on les a inoculés dans un tube indicateur de croissance de MGIT™ 960 dans les cultures STC-NRA en présence et en l'absence de PNB, un inhibiteur de la croissance du complexe *M. tuberculosis*. La croissance bactérienne est indiquée par la réduction de STC (modification de la couleur) et le comportement de NRA a été recherché pour la confirmation du complexe *M. tuberculosis*.
RÉSULTATS : La culture STC-NRA a été positive pour les bacilles acido-résistants dans 66/93 échantillons (71%), parmi lesquels 60/93 (64,5%) ont été identifiés comme positifs pour le complexe *M. tuberculosis* et 6/93 (6,5%) comme mycobactéries indéterminées. Le MGIT a mis en évidence complexe *M. tuberculosis* dans 59/93 cultures (63,4%). On a trouvé des contaminations dans 12/93 cultures STC-NRA vs. dans 29/93 cultures MGIT (31,2%). La durée moyenne avant la détection du complexe *M. tuberculosis* (TTD) a été de 14 jours avec STC-NRA vs. 7 jours avec MGIT.
CONCLUSION : La méthode STC-NRA est sensible pour la détection du complexe *M. tuberculosis* dans l'expectoration. Le TTD augmente avec la durée du traitement TB, ce qui suggère la valeur de cette méthode pour le suivi du succès du traitement. La méthode est simple, peu coûteuse et, à l'opposé de MGIT, n'exige pas un équipement technique, ce qui, en même temps que les caractéristiques préliminaires de performance, justifie une évaluation complémentaire dans des études plus importantes.

RESUMEN

MARCO DE REFERENCIA: Mbeya, Tanzania.
OBJETIVO: Desarrollar un nuevo método de cultivo en medio líquido para detectar el complejo *M. tuberculosis* (MTC) en esputo usando el 2,3-diphenyl-5-thienyl-(2)-tetrazolium (STC), el ensayo de reducción de nitratos (NRA) y el ácido para-nitrobenzoico (PNB).
MÉTODO: Noventa y tres muestras de esputo colectadas de 18 pacientes con tuberculosis fueron decontaminadas con NALC-NaOH e inoculados en MGIT™ 960 y en cultivo STC-NRA en presencia y ausencia de PNB, un inhibidor de crecimiento de MTC. La reducción de STC (cambio de color) indicó el crecimiento de micobacterias, y el momento para realizar la reacción de reducción de nitratos (NRA) y confirmación de MTC.
RESULTADOS: Los cultivos STC-NRA fueron positivos para bacilos ácido alcohol resistentes en 66/93 (71%), de los cuales 60/93 (64,5%) fueron identificados como MTC y 6/93 (6,5%) como micobacterias de especie no determinada. MGIT aisló MTC en 59/93 (63,4%) cultivos. La contaminación en cultivo STC-NRA fue 12/93 (13%) y 29/93 (31,2%) en cultivo MGIT. El tiempo promedio para detectar (TTD) MTBC fue 14 días en STC-NRA, y 7 días en MGIT.
CONCLUSIÓN: El método STC-NRA es sensible para la detección de MTC a partir de esputo. El TTD incrementó con la duración del tratamiento, sugiriendo la utilidad de este método para monitorear la respuesta al tratamiento. El método es simple, económico y a diferencia del cultivo MGIT no requiere equipos, lo que junto con las características preliminares de rendimiento justifica la ejecución de mayores estudios.

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PLOS | NEGLECTED TROPICAL DISEASES

Seroprevalence of *Alphavirus* Antibodies in a Cross-Sectional Study in Southwestern Tanzania Suggests Endemic Circulation of Chikungunya

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Abstract

Background: To date, *Alphavirus* infections and their most prominent member, chikungunya fever, a viral disease which first became apparent in Tanzania in 1953, have been very little investigated in regions without epidemic occurrence. Few data exist on burden of disease and socio-economic and environmental covariates disposing to infection.

Methods: A cross-sectional seroprevalence study was undertaken in 1,215 persons from Mbeya region, South-Western Tanzania, to determine the seroprevalence of anti-*Alphavirus* IgG antibodies, and to investigate associated risk factors.

Results: 18% of 1,215 samples were positive for *Alphavirus* IgG. Seropositivity was associated with participant age, low to intermediate elevation, flat terrain and with IgG positivity for Rift Valley fever, *Flaviviridae*, and rickettsiae of the spotted fever group. When comparing the geographical distribution of *Alphavirus* seropositivity to that of Rift Valley fever, it was obvious that *Alphaviruses* had spread more widely throughout the study area, while Rift Valley fever was concentrated along the shore of Lake Malawi.

Conclusion: *Alphavirus* infections may contribute significantly to the febrile disease burden in the study area, and are associated with several arthropod-borne infections. Their spread seems only limited by factors affecting mosquitoes, and seems less restricted than that of Rift Valley fever.

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Introduction

Alphaviruses form a genus in the family *Togaviridae*. About 40 different viruses including type and sub-type viruses are members of the genus. Among them are major human pathogens such as chikungunya virus (CHIKV) and viruses of veterinary importance, e.g. Venezuelan equine encephalitis virus. The currently most important *Alphavirus* of human pathogenicity is CHIKV, which causes significant morbidity and economic losses [1]. Although it has been described and isolated first in 1953 from a febrile person in Tanzania, East Africa [2], currently only few data on the distribution and medical importance of CHIKV and other *Alphaviruses* in Africa are available.

Since the 1960s, especially CHIKV was repeatedly isolated throughout African and Asian countries [3], and small outbreaks were frequently reported. The virus gained notoriety, when in the years 2004–2007 an outbreak was noticed of so far unknown dimension. Starting in Kenya, a severe epidemic hit the islands of the Indian Ocean in 2005/2006, with nearly 280.000 people infected on the island of La Reunion alone [1,4,5]. Transmission to the Indian sub-continent resulted in chikungunya fever in an estimated 1.3 million people [6]. The enormous scientific interest in this outbreak led to several new findings concerning viral molecular biology and ecology [3,7–9]. Investigations regarding the climatic conditions before the outbreak revealed unusual warm and dry conditions along the Kenyan coast in 2004 [10,11]. Infrequent replenishment of domestic water stores due to these dry conditions may have facilitated the transmission of the virus.

Despite this increased research interest, the role of CHIKV as well as other *Alphaviruses* in endemic regions, especially in sub-Saharan Africa, remains unclear. Recent studies concentrated mainly on areas of the latest CHIKV pandemic. The disease burden and the epidemiology in local populations not affected by the devastating outbreak in 2004–2007 is largely unknown. In a

Author Summary

The origin of febrile disease is often difficult to diagnose. In tropical countries, viral infections that are transmitted by arthropods include, among others, *Alphavirus* infections (e.g. chikungunya fever), dengue, West Nile, Yellow Fever and Rift Valley fever. In malaria endemic areas, these diseases are often mis-diagnosed and treated as malaria. Our study examined serum samples from 1,215 participants of a population survey from the Mbeya region, south-western Tanzania, for antibodies against *Alphaviruses* of the Semliki forest group as a sign of past infection. We found 18% of study participants positive, a surprisingly high number which points to a hitherto undetected circulation of *Alphaviruses* in the region. Among examined risk factors, even terrain, low to moderate elevation and participant age were associated with antibody positivity. Comparison with the distribution of Rift Valley fever seropositivity showed that *Alphaviruses* are more widely distributed throughout the study area, while Rift Valley fever seems to occur in a limited area close to Lake Malawi only.

small study in Guinea, arboviruses as causative agent for febrile disease were identified by neutralization assays in 63% of 47 patients [12]. 17% of these had acute CHIKV infections. In a clinical study conducted in Northern Tanzania with 870 febrile patients, PCR-confirmed acute CHIKV infections were diagnosed in 7.9% of all cases [13]. However, surveillance of other *Alphaviruses* is even less developed as most of these studies are targeting CHIKV using PCR. A serosurvey in rural Kenya revealed a seropositivity prevalence of 34% for anti-*Alphavirus* IgG, which was not associated with age, indicating frequently occurring smaller epidemics rather than endemic cycling [14]. Although CHIKV is expected as the main pathogen, other *Alphaviruses* cannot be excluded since a broadly cross-reactive ELISA was used.

With the recent outbreak of CHIKV in Italy, and detection of autochthonous transmission in southern France, it is clear that *Alphaviruses* and especially CHIKV have the potential to become endemic in areas in Europe where *Aedes albopictus* is already established [15,16].

In this study we aimed to assess the epidemiological patterns of *Alphavirus* infections in the Mbeya Region in Tanzania, by measuring seroprevalence in 1215 individuals participating in an epidemiological survey in the Region. This region was not affected by the 2004–2007 outbreak, and diagnosis or laboratory verification of acute chikungunya fever or other *Alphavirus* infection does not occur locally. The survey gave us the opportunity to study the role of this pathogen genus and its dependence on certain social and ecological factors in an endemic transmission cycle in a typical local setting.

Materials and Methods

Ethics statement

Both EMINI and this sub-study were approved by local and national Tanzanian ethics committees. Each EMINI participant had provided written informed consent before enrolment, with parents consenting for their children.

Study population and the EMINI survey

The EMINI population survey had the objective to create the infrastructure to **E**valuate and **M**onitor the **I**mpact of **N**ew **I**nterventions in the Mbeya Region of south-western Tanzania. Financed by the European Union over five years (2006 to 2011), the strengthening of the local health infrastructure and the establishment of a cohort which could be followed up on an annual basis created a platform on which the impact of improved health care infrastructure and new interventions could be monitored and evaluated. Embedded into the EMINI project were several focused studies such as this sub-study, which determined seroprevalences for a number of tropical arthropod-borne diseases.

In preparation of the EMINI survey, a census of the entire population in nine geographically distinct and ecologically different sites of the Mbeya Region was carried out. Study sites were selected to reflect the wide range of different conditions within the region in terms of elevation, population density and development (urban versus rural). Basic information regarding the households and their inhabitants was collected and all household positions were recorded with handheld GPS receivers. Ten percent of the surveyed households were then chosen by geographically stratified random selection for inclusion into the EMINI survey, to obtain a representative sample of the population from each site. The resulting EMINI cohort included all consenting participants of 4,283 households. Over the following five years annual visits at the same time of the year were conducted, during which structured interviews with all household members were performed, and blood, urine and stool specimens collected.

For this sub-study, we stratified the 17,872 participants, who had provided a blood sample during the second EMINI survey in 2007/2008, by age, gender, altitude of residence and ownership of domestic mammals. To be able to assess factors of interest that were identified in the literature but might have been underrepresented in the study population, we employed disproportionate random sampling with equal participant numbers for each stratum to identify 1.226 samples from participants above the age of 5 years to be tested for IgG antibodies against *Alphaviruses* and other tropical arthropod-borne diseases.

Socio-economic status

To characterize the socio-economic situation of each household, the head of each household was asked for the following information during each annual EMINI visit: Presence/absence of different items in the household (clock or watch, radio, television, mobile telephone, refrigerator, hand cart, bicycle, motor cycle, car, savings account), sources of energy and drinking water, materials used to build the main house, number of persons per room in the household and availability and type of latrine used. Based on the provided information, a socio-economic-status (SES) score was established, using a modified method originally proposed by Filmer and Pritchett which has frequently been employed to characterize the SES of people living in developing countries [17–19].

Environmental data

Population- and livestock-densities were calculated using data and household positions collected during the initial population census. Elevation data were retrieved from the NASA Shuttle Radar Topography Mission (SRTM) global digital elevation model, version 2.1 [20,21].

Land surface temperature (LST) and vegetation cover (EVI = enhanced vegetation index) data for the years 2003 to 2008 were retrieved from NASA's Moderate-resolution Imaging Spectroradiometer (MODIS) Terra mission which "are distributed by the Land Processes Distributed Active Archive Center (LP DAAC),

located at the U.S. Geological Survey (USGS) Earth Resources Observation and Science (EROS) Center (lpdaac.usgs.gov)" [22]. These data were used to produce long-term averages of day and night LST and EVI.

Population-, household-, and livestock-densities, LST, EVI, and elevation data were averaged for a buffer area within 1000 meter radius around each household in order to characterize the ecological situation around the household. This approach was preferred to using the respective spot values at the household position, because spot data are more prone to random error than averages for a wider area.

Serology

Detection of anti-*Alphavirus* IgG, anti-Yellow fever virus IgG, anti-dengue 1–4 virus IgG, and anti-West Nile virus IgG on bio-banked samples were performed as described previously for Rift Valley fever virus (RVfV) IgG [23]. A commercially available biochip (Euroimmun, Lübeck, Germany), containing infected and non-infected Vero E6 cells or only non-infected Vero E6 cells (negative control), was used for indirect immunfluorescence testing (IIFT), following a standard protocol. All serum samples were heat-inactivated and diluted tenfold prior to testing. Further dilutions of positive sera were carried out in the range of 1:20 to 1:640. A rabbit anti-human IgG FITC-labelled antibody (DAKO, Hamburg, Germany) served as conjugate. To decrease the known subjectivity of reading IIFT results to the best objective level, fluorescence microscopy was carried out independently by two experienced observers. In case of discrepancies (positive vs. negative; difference >1 titer step) the testing was repeated. A sample was classified as positive, if at a screening dilution of 1:20 a typical fine granular cytoplasmatic fluorescence was detected in around 20% of the cells on the positive field of the biochip with a typical location and morphology of infected cells, while no signal was detectable in the negative field. IIFT was repeated in case of indeterminate results, i.e. in cases where samples differed clearly from the negative control but did not match the criterion "positive". Ultimately, 1,215 definitive results were available from the selected samples.

Testing for *P. falciparum* malaria

Fresh EDTA-blood was used for malaria testing using a rapid test (ICT, South Africa) for each participant.

Data analysis

Stata statistics software (version 12, Statacorp, College Station, TX, USA) was used for all statistical analyses, and Manifold System 8.0 Professional Edition (Manifold Net Ltd, Carson City, NV) was used for processing of geographical data and to produce maps. In order to identify possible risk factors for anti-*Alphavirus* IgG positivity, we analysed seropositivity as the binary outcome in uni- and multi-variable Poisson regression models with robust (or Huber-White) variance estimates adjusted for household clustering [24,25]. Initial uni-variable models for all factors that we deemed as possibly related to CHIKV infection were used to identify variables with a uni-variable p-value $<=0.1$ for further multi-variable evaluation. Stepwise backward and forward regression, the Akaike and Bayes information criterion and various assessments of model-fit were used to identify the best multi-variable model, where only variables with a multi-variable p-value <0.1 were retained.

Associations of anti-*Alphavirus* IgG positivity with other diseases were assessed in uni-variable Poisson regression models with anti-*Alphavirus* IgG as the binary outcome, and the respective disease as the only predictor variable. In addition we ran the same models adjusted for those risk factors that had been retained in the above described multi-variable models regarding risk factors for anti-*Alphavirus* IgG positivity.

In the analysis, positivity for at least one of dengue, West Nile and Yellow fever antibodies, was categorized as "*Flavivirus* IgG" positive.

Results

219 of 1,215 (18.0%) samples reacted positive for anti-*Alphavirus* IgG. The estimated overall population prevalence, predicted from our stratified sample by direct extrapolation, is 11.8% for the population of our 9 sites (fig. 1).

Seropositivity increased with participant age, both in uni- and in multi-variable analysis (table 1; prevalence ratio (PR) for a 10-year increase in multi-variable analysis: 1.26, 95% confidence interval (CI) 1.20 to 1.32, $p<0.001$). Gender was not significantly associated with seropositivity.

We found a significant association of anti-*Alphavirus* IgG status with elevation above sea level, with significantly higher seroprevalence in the strata below 1,198 m, both in uni-variable and multi-variable analysis (fig. 2). The median elevation of the Kyela site is 487 m (Interquartile range IQR 483 m–514 m), and that of Igurusi is 1,193 m (IQR 1,156–1,205 m). Not only elevation, but also slope of the terrain was negatively associated with seropositivity in uni- and multi-variable analysis, even when adjusted for age and elevation (PR 0.86 per degree, 95% CI 0.77 to 0.95, $p = 0.004$), with the highest anti-*Alphavirus* IgG prevalence occurring on terrain with a slope of less than $\sim 1.6^{\circ}$ (fig. 2).

Several social, economic and behavioural factors showed significant association in uni-variable analysis but were rendered non-significant in multi-variable analysis when adjusting for age, elevation and slope of terrain. Factors associated with higher anti-*Alphavirus* IgG prevalence in uni-variable analysis included a lower socio-economic status, lower population density, higher vegetation density and higher land surface temperatures, especially night temperatures. Also, bed net ownership and higher frequency of use, which occurred in areas with higher mosquito burdens, were associated with a higher seropositivity in uni-variable analysis. Anti-*Alphavirus* IgG status was not associated with animal ownership, including cattle, sheep, goats and chicken (data not shown).

Next, we analysed correlations between anti-*Alphavirus* IgG status and other infectious diseases throughout the survey. Uni-variable analyses showed significant positive associations of anti-*Alphavirus* IgG with *P. falciparum* malaria RDT positivity, antibody positivity for spotted fever group rickettsiae (SFG) and Rift Valley fever virus (RVFV) [23], and any of the tested *Flaviviridae* (table 2). In separate models for each of these pathogens, that were adjusted for age, elevation and slope of terrain, a significant positive association was retained for SFG IgG, *Flavivirus* IgG and RVFV IgG, while the association with *P. falciparum* disappeared. A comparison of the spatial distribution of RVFV IgG and anti-*Alphavirus* IgG shows that for both viruses, Kyela site has the highest seroprevalences, but a wider occurrence is seen for anti-*Alphavirus* IgG when compared to RVFV IgG (fig. 1).

HIV status was unrelated with individual anti-*Alphavirus* IgG status in uni- and multi-variable analysis (data not shown).

Discussion

In the current study we present high rates of IgG antibodies against an *Alphavirus*. Cross reactions mainly occur in IFAT between antibodies against CHIKV and other viruses of the

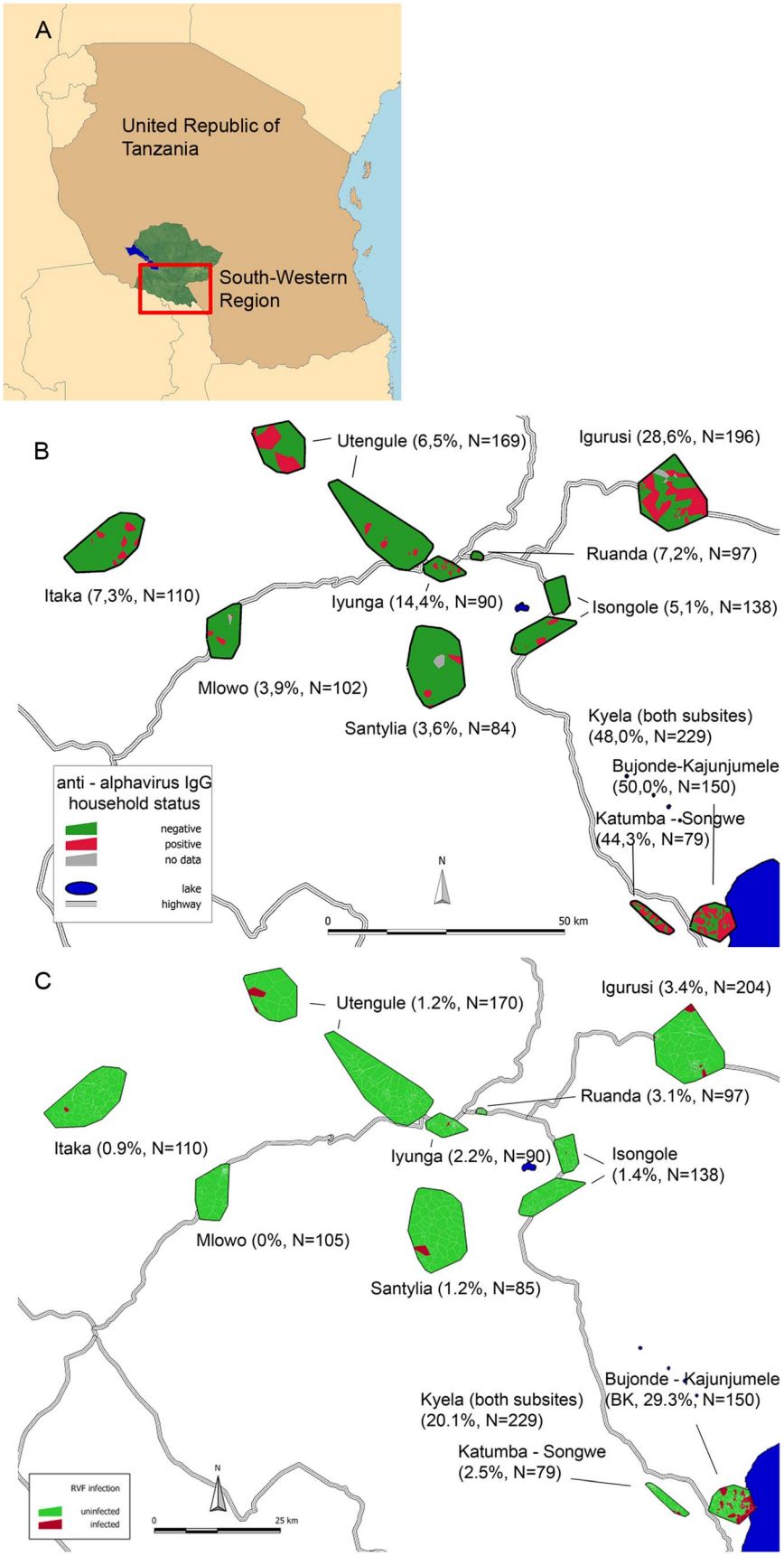
A
United Republic of Tanzania
South-Western Region
B
Utengule (6,5%, N=169)
Igurusi (28,6%, N=196)
Ruanda (7,2%, N=97)
Itaka (7,3%, N=110)
Iyunga (14,4%, N=90)
Isongole (5,1%, N=138)
Mlowo (3,9%, N=102)
Santylia (3,6%, N=84)
Kyela (both subsites) (48,0%, N=229)
Bujonde-Kajunjumele (50,0%, N=150)
Katumba - Songwe (44,3%, N=79)
anti - alphavirus IgG household status
negative
positive
no data
lake
highway
N
0
50 km
C
Utengule (1.2%, N=170)
Igurusi (3.4%, N=204)
Ruanda (3.1%, N=97)
Itaka (0.9%, N=110)
Iyunga (2.2%, N=90)
Isongole (1.4%, N=138)
Mlowo (0%, N=105)
Santylia (1.2%, N=85)
Bujonde - Kajunjumele (BK, 29.3%, N=150)
Kyela (both subsites) (20.1%, N=229)
Katumba - Songwe (2.5%, N=79)
RVF infection
uninfected
infected
N
0
25 km

Figure 1. Location of Households with positive participants for anti-*Alphavirus* IgG and Rift Valley fever virus IgG. Localisation of the study area in Tanzania (A). Location of households with (B) *Alphavirus* IgG-positive and (C) Rift Valley fever virus IgG-positive participants displayed as Voronoi polygons, with every polygon representing one household. Percent IgG-positives and total N examined in the site are displayed with site name. Households with one or more individuals positive for *Alphavirus* IgG are marked in red, all others in green. For Kyela site, both subsites Bujonde-Kajunjumele and Katumba-Songwe are displayed. Map created by use of Manifold System 8.0 software. (C) reproduced from [23] under creative commons license.
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Semliki-Forest virus complex of *Alphaviruses*, while cross reactivities against the Venezuelan equine, the Eastern equine and the Western equine encephalitis group are rare and low ($\geq$4 titer steps; Dobler, unpublished observations). Therefore we assume that cross reactions may mainly occur between Semliki Forest complex viruses like O′nyong nyong virus or Semliki Forest virus. Other non-African Semliki-Forest virus complex viruses, like Ross River virus or Mayaro virus do not seem to be responsible for the antibodies as the inhabitants of the areas tested did not leave the region. However, we cannot exclude that a so far unknown *Alphavirus* of the Semliki Forest virus complex is circulating and may cause infection with or without clinical symptoms. The question can only be answered by virus detection by isolation or molecular detection and characterization of genome parts.

This analysis of the seroprevalence for *Alphaviruses* adds to the picture of arthropod-borne infectious diseases in our study population. Together with previous reports on RVFV, rickettsiae of the typhus group and spotted fever group, we are demonstrating comparably high seroprevalences which could be caused by considerable exposure of the population to arthropod-borne infections other than malaria [23,26,27]. Akin to RVFV, a near-linear correlation of anti-*Alphavirus* IgG prevalence with age suggests endemic exposure rather than single or few epidemic events.

Acute *Alphavirus* infections such as chikungunya fever are neither known nor regularly diagnosed in the health facilities in the region, and might be overlooked by medical staff as a possible causative agent for febrile illness, leading to presentation at the health facility. Febrile disease in the area is mostly regarded as malaria by treating clinicians, despite the fact that our survey showed a marked reduction of *P. falciparum* infection since the introduction of artemether - lumefantrine as first line therapy in 2006 [28]. Therefore, the awareness for zoonoses as a possible underlying cause of febrile illness should be increased.

Our analysis shows that anti-*Alphavirus* IgG prevalence is associated with geographical features related to favourable mosquito breeding conditions. These include low to moderate elevations and flat terrain, which disposes to the formation of surface water collections [4].

Climate has been consistently pointed out as one of the major determinants for the distribution of vector borne diseases. Although lower larval rearing temperatures result in increased likelihood of adult female mosquitoes becoming infected with CHIKV or other arboviruses in laboratory experiments [29,30], it is higher temperatures which are generally linked to more efficient disease transmission in laboratory and epidemiological investigations [31]. However, the temperature variables examined here dropped out of the multi-variable model due to lack of multi-variable significance, with the elevation variable obviously producing a better fit than the satellite-measured land surface temperatures. In La Reunion, the spread of *Ae. albopictus* has been found to be limited to elevations $<$1200 m in summer [32]; in Gharwal/India, spread was limited to $<$1.400 m. This corresponds well with the drop in seroprevalence in strata above 1197 m (table 1). We still assume that temperature is the causal limiting factor in higher elevations, not other elevation-dependent factors such as radiation or atmospheric pressure. It should thus be kept in mind that our elevation results should not be generalized to predict infection risk in other climatic settings.

Comparisons with our data on spread of other mosquito-borne infections, namely RVF, *Flaviviridae* and *P. falciparum* malaria, and the tick-borne spotted fever group (SFG) rickettsiae, produce interesting findings. The uni-variable association of *P. falciparum* to anti-*Alphavirus* IgG disappears when adjusting for age, elevation and slope, suggesting that this association was due to factors supporting the breeding of the different mosquito vectors alike. We did not test to distinguish between anti-CHIKV IgG and o′nyong′nyong virus IgG due to lack of capacities to perform the neutralization test, so it is possible that the seroprevalence is caused by more than one virus.

A similar association exists between bednet ownership and anti-*Alphavirus* IgG. Bednet ownership is not homogenous over the study area, but more frequent in areas of higher malaria transmission, which in our data are characterised by low elevation and even terrain, favouring standing surface water as mosquito breeding grounds. This not only supports *Anopheles* but also other mosquito species, so the use of bednets can be seen as a proxy for general abundance of mosquitoes – hence the positive association in uni-variable analysis. When corrected for elevation and slope of terrain, the association with bednets disappeared – showing that in malaria endemic areas, bednet ownership neither favours nor protects against *Alphavirus* infection. This may point towards a diurnally active vector such as *Aedes* spp., against which bednets do not protect.

The association of anti-*Alphavirus* IgG with RVFV and *Flavivirus* IgG, viruses sharing *Aedes* spp. as vector, is however retained in multivariable analysis. This shows that in addition to age, elevation and slope, additional relevant factors still influence the spread of these *Aedes* – borne infections which remain to be identified. Others have found higher seroprevalences in Cameroonians living under corrugated iron roofs vs. thatched grass roofs; furthermore, living in rural areas was associated with higher seroprevalences [33].

SFG rickettsiae and *Alphaviruses* are transmitted by completely different vectors (cattle ticks and mosquitoes respectively), therefore the reason for the observed association is not clear. Rural living conditions, defined by low population density and long distances to roads, was a risk factor in our analysis of SFG rickettsia IgG [27]. Others authors also found this to be a risk factor for anti-*Alphavirus* IgG [33], so it is possible that rural conditions are the factor which increases the risk for both of these diseases which do not have much else in common.

Interesting are the differences in geographical spread of anti-*Alphavirus* IgG versus RVFV IgG in our population. Anti-*Alphavirus* IgG is more evenly distributed in the two Kyela subsites, and is also common in other sites. RVFV IgG on the other hand concentrates along the shore of Lake Malawi and nearby watercourses. The preferred occurrence of RVFV along water

Table 1. Association of anti-*Alphavirus* IgG status with environmental and socio-economic factors.

				uni-variable[1]			multi-variable[2]		
Covariate	**stratum**	**N**	**% pos.**	**PR**	**95% conf.int.**	**p-val**	**PR**	**95%conf.int.**	**p-val**
Age									
	per 10 years	1215		1.24	(1.18 to 1.30)	<0.001	1.26	(1.20 to 1.32)	<0.001
Slope									
	per degree	1215		0.58	(0.50 to 0.66)	<0.001	0.86	(0.77 to 0.95)	0.004
Elevation (m)									
	479.1-	122	50.0	1	-	-	1	-	-
	487.5-	122	43.4	0.87	(0.66 to 1.14)	0.317	1.09	(0.85 to 1.41)	0.504
	973.7-	118	33.9	0.68	(0.49 to 0.94)	0.019	0.73	(0.54 to 0.98)	0.038
	1197.8-	120	15.8	0.32	(0.20 to 0.51)	<0.001	0.41	(0.25 to 0.66)	<0.001
	1290.9-	123	4.9	0.10	(0.04 to 0.22)	<0.001	0.16	(0.07 to 0.37)	<0.001
	1491.4-	120	4.2	0.08	(0.03 to 0.20)	<0.001	0.13	(0.05 to 0.34)	<0.001
	1578.0-	122	10.7	0.21	(0.12 to 0.37)	<0.001	0.27	(0.15 to 0.47)	<0.001
	1612.8-	123	9.8	0.20	(0.11 to 0.35)	<0.001	0.23	(0.13 to 0.40)	<0.001
	1724.5-	123	5.7	0.11	(0.05 to 0.24)	<0.001	0.19	(0.08 to 0.43)	<0.001
	2002.8-	122	2.5	0.05	(0.02 to 0.15)	<0.001	0.10	(0.03 to 0.34)	<0.001
Gender							*This and the following variables were not included into multi-variable analysis due to lack of multi-variable significance.*		
	female	667	17.1	1	-	-			
	male	540	19.3	1.13	(0.89 to 1.42)	0.318			
	missing data	8	12.5	0.73	(0.12 to 4.61)	0.739			
SES Rank									
	per unit	1215		0.90	(0.86 to 0.94)	<0.001			
Bednet owned									
	No	692	10.4	1	-	-			
	Yes	523	28.1	2.70	(2.07 to 3.53)	<0.001			
Frequency of bednet use									
	Never	694	10.2	1	-	-			
	Sometimes	55	16.4	1.60	(0.84 to 3.05)	0.153			
	Most times	21	23.8	2.33	(1.10 to 4.94)	0.028			
	Always	443	30.0	2.93	(2.25 to 3.84)	<0.001			
	missing data	2	50.0	4.89	(1.20 to 19.87)	0.027			
Persons/km^2									
	per unit	1215		0.92	(0.87 to 0.97)	0.002			

Table 1. Cont.

				uni-variable[1]			multi-variable[2]		
Covariate	**stratum**	**N**	**% pos.**	**PR**	**95% conf.int.**	**p-val**	**PR**	**95%conf.int.**	**p-val**
Enhanced Vegetation Index (Max.)									
	per 0,1 units	1215		2.03	(1.56 to 2.65)	<0.001			
Enhanced Vegetation Index (Avg.)									
	per 0,1 units	1215		2.53	(2.04 to 3.14)	<0.001			
Enhanced Vegetation Index (Min.)									
	per 0,1 units	1215		2.49	(1.92 to 3.23)	<0.001			
Average Land Surface Temperature									
	per 10°	1215		1.73	(1.02 to 2.93)	0.043			
Night Land Surface Temperature									
	per 10°	1215		9.70	(6.91 to 13.60)	<0.001			
Rainfall									
	per unit	1215		1.01	(1.01 to 1.01)	<0.001			

Results of Poisson regression models adjusted for household clustering using robust variance estimates.

N= number of observations; % pos. = percent anti-*Alphavirus* IgG positive in stratum; PR = Prevalence ratio; 95% conf.int = 95% confidence interval; SES rank = rank (from 0 for lowest to 10 for highest) according to socio-economic score.

1: results of separate models for each of the below covariates.

2: multivariable model including only age, elevation and slope of terrain as covariates.

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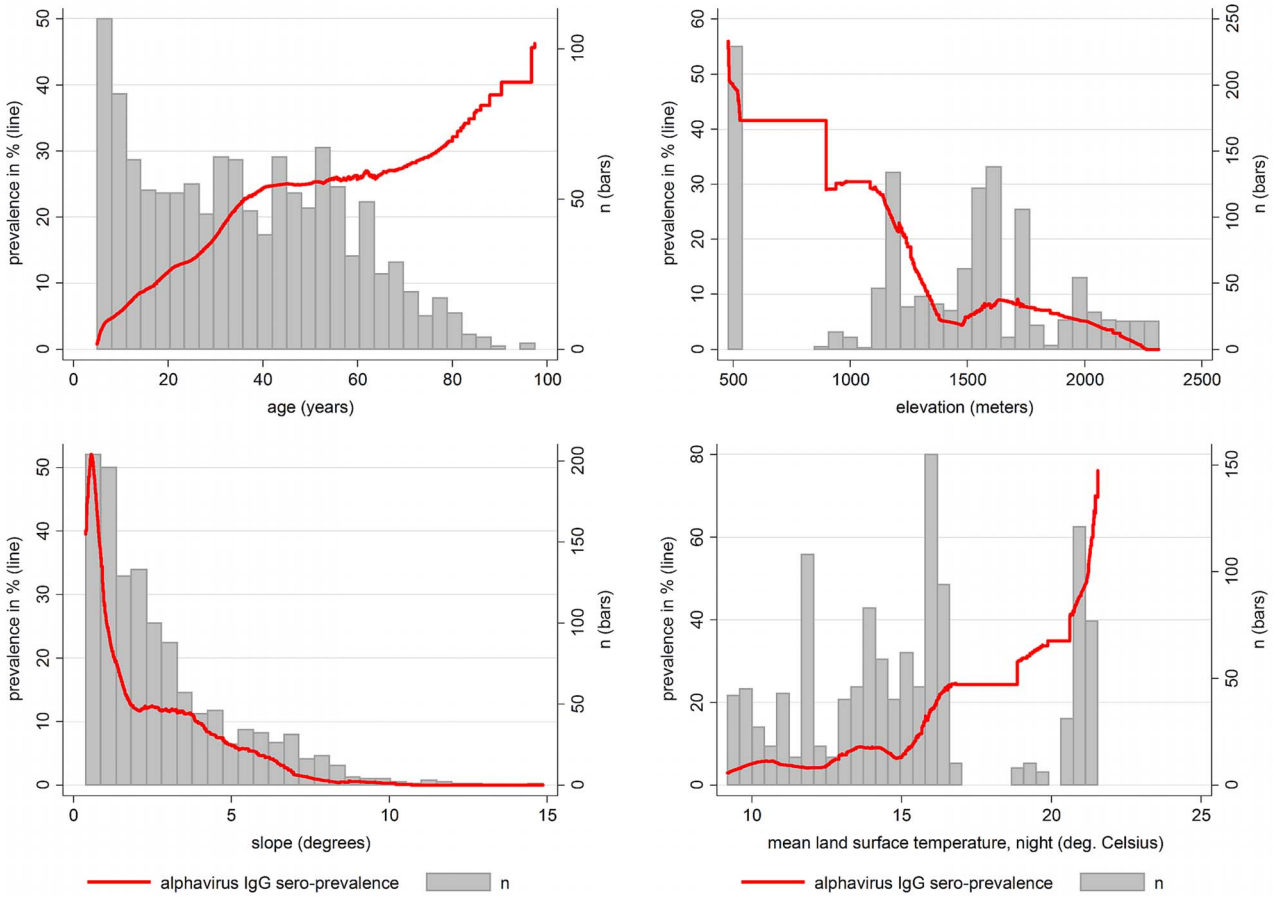

Figure 2. Anti-*Alphavirus* IgG prevalence in association with age, elevation, slope and land surface temperature. Lowess smoothed plots of anti-ChikV IgG-seroposivitivity over age, elevation, slope of terrain, and land surface temperature during the night. Red line: anti-*Alphavirus* seroprevalence. Grey bars: number of observations in stratum.
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bodies has been demonstrated in other settings as well [34], but does not seem to apply to anti-*Alphavirus* IgG positivity in our setting. This observation may imply that the vectors of RVFV in Kyela region are floodwater mosquito species and therefore need the shore of Lake Malawi, whilst the vectors of the *Alphavirus* may show an anthropophilic behaviour. The strong affinity to water, which applies for RVFV, but not for the *Alphavirus*, may also be related to a higher density of cattle as RVFV reservoir hosts along the water, or with the transovarial transmission of RVFV in diapausing *Aedes* mosquitoes along waterbodies [35]. It is also possible that RVFV requires a temperature optimum as suggested by our previous work, with a direct correlation with higher minimum temperatures, lower maximum temperatures, and positive influence of dense vegetation, conditions that are fulfilled mainly at the lakeshore [23]. The causative *Alphavirus*, despite probably limited to a smaller number of vector species compared to RVFV, seems to be less specific in terms of ecological conditions and seems to show a more anthropophilic behaviour, leading to a wider spread of the virus throughout the study area.

In summary, our data suggest that CHIKV or a closely related *Alphavirus* like o′nyong′nyong virus is circulating in the study area. If this virus causes disease, it could be an important cause of febrile illnesses in the region, and may be currently underdiagnosed. The linear relation of seropositivity to age suggests endemic rather than epidemic cycling, opposed to a study from Kenya where seropositivity was linked by the authors to epidemic exposure [14]. Kenya was reportedly affected by past CHIKV outbreaks, while there are no reports of outbreaks from our study area. A study from northern Tanzania reported acute CHIKV infection in 7.9% of febrile hospitalized patients, demonstrating that CHIKV circulates between epidemics in the country and may well be responsible for the seroprevalences in our study [13].

The power of our statements is further limited by the stratified nature of our study cohort, which results in prevalence levels slightly different from the general population. Further, the serological method used does not allow distinguishing between different *Alphavirus* species, and only gives information on cumulative lifetime infection risk. Therefore, prospective studies are needed to establish the rate of acute fever caused by CHIKV or other *Alphaviruses* in febrile patients. If the infecting *Alphavirus* is shown to be CHIKV or another *Alphavirus* of human medical importance these results should lead to a re-assessment of the local diagnostic algorithm for febrile illnesses, to take into account the endemic presence of the causative *Alphavirus(es)* in the area. These studies would also have to answer the question whether endemic strains do have a reduced pathogenicity, and have evaded detection by not causing the typical symptoms.

Table 2. Association of anti-*Alphavirus* IgG status with other infectious diseases.

				uni-variable[1]			adjusted[2]		
Covariate	**stratum**	**N**	**% pos.**	**PR**	**95% conf.int.** ‡	**p**	**PR**	**95%conf.int.**	**p**
SFG Rickettsiae IgG status									
	neg.	392	9.4	1	-	-	1	-	-
	pos.	823	22.1	2.34	(1.67 to 3.28)	<0.001	1.51	(1.11 to 2.06)	0.008
Rift Valley fever IgG status									
	neg.	1151	15.2	1	-	-	1	-	-
	pos.	62	71.0	4.67	(3.78 to 5.76)	<0.001	1.68	(1.25 to 2.25)	0.001
	miss.[3]	2	0.0	ND	-	-	ND	-	-
Any *Flavivirus* IgG status									
	neg.	1049	13.3	1	-	-	1	-	-
	pos.	161	49.1	3.68	(2.95 to 4.59)	<0.001	1.34	(1.06 to 1.70)	0.013
	miss.[3]	5	0.0	ND	-	-	ND	-	-
***P. falciparum* status**									
	neg.	1195	17.8	1	-	-	1	-	-
	pos.	18	33.3	1.87	(0.96 to 3.64)	0.065	1.01	(0.53 to 1.94)	0.970
	miss.[3]	2	0.0	ND	-	-	ND	-	-

Results of Poisson regression models adjusted for household clustering using robust variance estimates.

N = number of observations; % pos. = percent anti-*Alphavirus* IgG positive in stratum; PR = Prevalence ratio; 95% conf.int = 95% confidence interval; SFG: spotted fever group rickettsiae; IgG = Immunogolbulin G; ND = not done.

1: results of separate uni-variable models for each of the above infections.

2: results of separate multi-variable models for each of the above infections adjusted for age, elevation and slope of terrain as covariates.

3: 95% confidence interval and p-value not calculated due to lack of variability of the outcome variable in this stratum.

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Supporting Information

Strobe Checklist S1 The STROBE checklist for quality assurance in reporting of observational studies is attached.
(PDF)

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Author Contributions

Conceived and designed the experiments: GD ES TL MH NH. Performed the experiments: NW NEN PC LM IK. Analyzed the data: ES NH NW GD. Contributed reagents/materials/analysis tools: GD. Wrote the paper: NW PC ES TL NH.

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