Accelerating the development of an extended-specificity multiplex urine immunoassay for the diagnosis and serotyping of pneumococcal pneumonia in the high carriage and disease burden setting of Blantyre, Malawi

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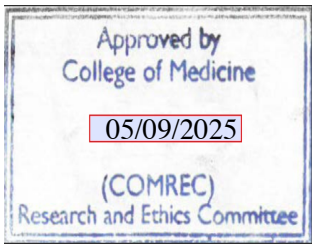

List of Abbreviations

Abbreviation	Term
24ssUAD	24-plex Serotype-specific urinary antigen detection
ARI	Acute Respiratory Infection
AUC	Area under the curve
ARGs	Antibiotic resistance genes
COMREC	College of Medicine Research and Ethics Committee
CRSU	Clinical Research Support Unit
GPCC	Gateway Primary Care Centre
LMICs	Low and Middle Income Countries
LSTM	Liverpool School of Tropical Medicine
MLW	Malawi Liverpool Wellcome Research Programme
NHC	Ndirande Health Centre
NHS	National Health Service
NPS	Nasopharyngeal Swab
PCVs	Pneumococcal conjugate vaccines
PERCH	Pneumonia Etiology Research for Child Health
QECH	Queen Elizabeth Central Hospital
QC	Quality control
ROC	Receiver Operating Characteristic
sSA	Sub Saharan Africa
ssUAD	Serotype-specific urinary antigen detection
SOPs	Standard Operating Procedures
UCL	University College London
UKHSA	UK Health Security Agency

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Executive Summary

Study type: This is a nested case-control sub-study within the multi-site, observational NP Resistome study (COMREC reference P.10/24-1200).

Problem: Pneumococcal pneumonia is a leading cause of morbidity and mortality among children under five, especially in sub-Saharan Africa. Accurate diagnosis remains a challenge due to the need for invasive specimen collection and poor sensitivity of standard culture-based diagnostic tests, particularly after antibiotic use. Serotype-specific urinary antigen detection (ssUAD) offers a promising, non-invasive alternative for serotype surveillance and diagnosis of pneumococcal disease. Serotype-specific identification provided by ssUAD is crucial for monitoring the impact of vaccines and informing public health interventions. The ssUAD test is a Luminex-based urine antigen capture assay developed by the UK Health Security Agency (UKHSA) that targets 24 pneumococcal serotypes, with good sensitivity and specificity among adults with community-acquired pneumonia in the UK. Further investigation is required to determine its ability/utility to identify invasive disease among children, particularly in settings like sub-Saharan Africa, where high rates of asymptomatic carriage may affect diagnostic accuracy.

Objectives

Broad:

To evaluate the performance of the ssUAD test in detecting pneumococcal carriage and distinguishing it from invasive disease among children under five years old in Blantyre, Malawi.

Specific:

1. To determine the prevalence of serotype-specific pneumococcal antigens in urine among children under five years of age with pneumonia compared to their healthy counterparts.
2. To characterise the association between the detection of serotype-specific antigens in urine and nasopharyngeal pneumococcal carriage among children.

3. To define revised ssUAD thresholds that may distinguish pneumococcal carriage from disease, to support field evaluations and inform diagnostic and surveillance strategies in Malawi and similar settings.

Methodology: We will test 350 existing urine samples that have already been collected from children as part of the NP Resistome study (Protocol v5.0 COMREC reference P.10/24-1200), including healthy children in the community, children with pneumonia in the community, and children hospitalised with pneumonia at the time of recruitment. Participants of the NP Resistome study will be recruited from Ndirande Health Centre (NHC), Gateway Primary Care Centre (GPCC), and Queen Elizabeth Central Hospital(QECH) in Blantyre, Malawi. Urine samples will be aliquoted into 1.8 mL cryotubes and stored at -80°C at Malawi Liverpool Wellcome Research Programme (MLW) laboratory in Malawi. Aliquots from each urine sample will be tested using the ssUAD in the UK, as the assay is not currently available in Malawi. However, we are working towards developing and evaluating the assay locally as part of future implementation efforts. Urinary detection of pneumococcal serotypes will be compared with both culture-based and metagenomic sequencing results from nasopharyngeal swab (NPS) samples taken as part of the main study.

Expected results and dissemination: This sub-study will generate key data on the prevalence of pneumococcal serotype-specific urinary antigens in children with pneumonia and healthy controls. It will improve understanding of ssUAD performance as a surveillance tool in this setting, help distinguish antigenuria due to carriage from disease, and refine diagnostic thresholds for use in high-carriage and disease-burden settings. Study findings will inform surveillance, pneumonia diagnostics, and vaccine impact assessments in LMICs. Results will be shared with COMREC, the Blantyre District Health Office, at scientific conferences, both local and international, and in peer-reviewed publications.

1.0 Background

Streptococcus pneumoniae (the pneumococcus) is a common commensal of the human nasopharynx, transmitted primarily through respiratory secretions[1]. While typically asymptomatic during carriage, it serves as a prerequisite for the development of invasive disease[2]. The pneumococcus causes a range of life-threatening infections, including pneumonia, meningitis, and bacteraemia, with a disproportionately high burden among children in sub-Saharan Africa (sSA)[3]. It remains a leading cause of childhood pneumonia worldwide. It contributes significantly to morbidity and mortality in children under five years of age, particularly in low- and middle-income countries (LMICs)[4,5]. Although the introduction of pneumococcal conjugate vaccines (PCVs) has significantly reduced the burden of vaccine-type disease, pneumonia caused by both vaccine and non-vaccine serotypes continues to present significant diagnostic challenges[6]. These challenges stem from the reliance on invasive specimen collection, such as blood or endotracheal aspirates and the limited availability of sensitive diagnostic tools in resource-limited settings. Conventional culture-based methods are further hampered by low sensitivity, especially when antibiotics have been administered before specimen collection[7]. These limitations undermine disease surveillance, leading to underestimation of the true burden of pneumococcal disease and the overall impact of vaccination programmes

Serotype-specific urinary antigen detection (ssUAD) has emerged as a promising non-invasive tool for diagnosing and serotyping pneumococcal pneumonia[8]. The ssUAD test is a 25-plex Luminex-based extended-range urine antigen capture assay developed by UKHSA, which has shown favourable results for diagnosing pneumococcal disease among adult patients in the UK. The 24-serotype-specific urine antigen detection (24ssUAD) assay can detect pneumococcal capsular polysaccharide antigens in urine, offering greater diagnostic sensitivity than the culture-based method[9]. The current version of ssUAD targets 24 pneumococcal serotypes and has a sensitivity of 96.2% and specificity of 89.9% among adults with community-acquired pneumonia or invasive pneumococcal disease in the UK[8]. However, there is limited data on the surveillance and diagnostic performance of ssUAD in high-carriage and disease-burden settings, such as Malawi, where asymptomatic or healthy children may also shed pneumococcal antigens in urine, making it more challenging to distinguish invasive disease. Unpublished data from our testing of the 24ssUAD assay on historical urine samples from Malawian children

with acute respiratory infection (ARI) indicate that pneumococcal antigens can be detected in urine, suggesting the assay's potential utility in such settings and highlighting the need for further evaluation to distinguish between carriage and disease. The data also revealed that pre-hospital antibiotic exposure significantly reduced the sensitivity of culture-based methods, decreasing pneumococcal detection rates from 81% to 21%, highlighting the potential diagnostic advantage of urine antigen assays in such contexts.

1.1. Literature Review

The accurate diagnosis of pneumococcal pneumonia remains a persistent challenge in both clinical and surveillance contexts, especially among young children in high-burden settings[10]. Several studies have demonstrated that reliance on conventional microbiological diagnostics, such as blood culture, results in significant under-detection of pneumococcal infections due to their low sensitivity and the frequent use of antibiotics before hospital admission[11,12]. In a systematic review by the Pneumonia Etiology Research for Child Health (PERCH) project, the limitations of blood culture sensitivity were particularly evident in children, where pathogen recovery was often below 10%[13].

These diagnostic limitations have prompted interest in antigen detection technologies, which are not reliant on viable organisms[14]. Among these, the ssUAD assay has gained prominence[8]. The ssUAD platform detects pneumococcal capsular polysaccharides in urine and allows for simultaneous serotyping of up to 24 pneumococcal serotypes using a multiplex Luminex-based platform[8,15]. Studies conducted in adult populations in high-income countries have reported high sensitivity and specificity for the assay, particularly in cases of community-acquired pneumonia[16].

A critical advantage of ssUAD is its performance in patients who have received antibiotics. Multiple studies have reported that urinary antigen tests retain high diagnostic sensitivity even after antibiotic treatment, in contrast to sputum and blood cultures, which show significantly reduced positivity. Similarly, in studies involving hospitalised adults, both the BinaxNOW ICT and ssUAD assays were able to detect pneumococcal antigens in urine despite negative cultures due to prior antibiotic exposure. However, ssUAD appears to offer superior specificity and serotype information. In contrast, BinaxNOW does not provide serotype information and has

been associated with false positives, particularly in settings with high pneumococcal carriage[18]. This property makes ssUAD a particularly attractive diagnostic tool in LMICs, where empiric antibiotic treatment is often initiated before specimen collection.

Available evidence primarily comes from adult populations where nasopharyngeal colonisation is relatively uncommon and less likely to interfere with urine-based antigen detection[19]. In contrast, children in sSA, including Malawi, often exhibit high nasopharyngeal carriage rates[20,21], which may make it difficult to distinguish between antigenuria due to invasive disease and antigen spillover from asymptomatic carriage.

Limited data exist on the specificity of ssUAD in high-carriage paediatric populations, particularly in low-resource settings where distinguishing between pneumococcal disease and antigenuria from asymptomatic carriage remains challenging. As such, there is an urgent need for context-specific data to define serotype-specific thresholds that can accurately differentiate carriage from disease. Addressing this gap is critical for the optimal use of ssUAD in clinical diagnostics, disease surveillance, and vaccine impact evaluations in high-burden LMIC settings.

1.2. Rationale

There is a critical need to improve pneumococcal pneumonia diagnostics and surveillance in LMICs where high nasopharyngeal carriage rates and frequent antibiotic use limit the utility of traditional culture-based methods[22,23].The ssUAD assay has the potential to fill this gap, but its diagnostic thresholds must be tailored to high-burden settings. By evaluating the prevalence and distribution of serotype-specific urinary antigens in children with and without pneumonia, and assessing how nasopharyngeal carriage influences antigen detection, this study will generate the evidence necessary to define thresholds that could distinguish carriage from disease. These data will guide the use of ssUAD in diagnostics, surveillance, and vaccine impact studies in LMICs.

1.2.1. Summary of NP Resistome study

This parent study is a multi-site observational extended observational case–control study with a preliminary cohort component designed to examine nasopharyngeal resistome (NPR) evolution following antibiotic selective pressure and healthcare exposure for pneumonia in children in Blantyre, and whether this is associated with adverse health outcomes. The

nasopharynx is a reservoir for bacteria that cause pneumonia in children, and their associated antimicrobial resistance. The NPR encompasses all the genetic determinants of antibiotic resistance found in this microbial niche. The study will recruit 350 children aged 12–24 months in four groups over an 18-month period; healthy children in the community, children with pneumonia in the community, children hospitalised with pneumonia and children re-hospitalised with pneumonia. The study will be conducted at Ndirande Community Health Centre and Queen Elizabeth Central Hospital. Both nasopharyngeal swabs (NPS) and urine samples will be taken at recruitment; only NPS will be taken at follow-up study visits. NPS will undergo metagenomic sequencing to identify the nasopharyngeal microbiome and NPR, and a viral PCR to identify viral pathogens associated with acute respiratory infections requiring hospitalisation in this age group; urine samples will be used for an antibiotic bio-assay. Demographics, co-morbidities, health outcomes and antibiotic exposure will be recorded at recruitment for all participants, and subsequent study visits where applicable.

The diversity and relative abundance of antibiotic resistance genes (ARGs) in the NPR will be compared between the study groups to determine whether a hospital-acquired NPR persists after discharge, and is associated with adverse health outcomes, particularly recurrent hospitalisation. The NP Resistome study will also provide contemporary data on AMR carriage in the community, hospital admission and re-admission rates with pneumonia, and antibiotic use patterns.

1.3. Hypothesis

We hypothesise that the ssUAD assay will show high sensitivity for detecting pneumococcal serotype-specific antigens in children with pneumonia, but background antigenuria due to asymptomatic carriage will necessitate revised thresholds for accurate disease attribution in this high-burden setting.

1.4. Study Objectives

1.4.1. Broad objective

To evaluate the performance of the ssUAD test in detecting pneumococcal carriage and distinguishing it from invasive disease among children aged 12-24 months with pneumonia, compared to healthy children, in Blantyre, Malawi.

1.4.2. Specific objectives

1. Determine the prevalence of serotype-specific pneumococcal antigens in urine among children under five years of age with pneumonia compared to healthy counterparts.

2. To characterise the association between the detection of serotype-specific antigens in urine and nasopharyngeal pneumococcal carriage among children.

3. To define revised ssUAD thresholds that distinguish pneumococcal carriage from disease, to support field evaluations and inform diagnostic and surveillance strategies in Malawi and similar settings.

2.0 Methodology

2.1. Study Design and Setting:

This case-control sub-study is nested within the NP Resistome study (Protocol v5.0 COMREC reference P.10/24-1200), a prospective observational cohort conducted in Blantyre, Malawi. Recruitment will take place at Ndirande Health Centre (NHC), Gateway Primary Care Centre (GPCC), and Queen Elizabeth Central Hospital (QECH). The sub-study will involve the analysis of urine samples that have already been collected from children aged 12–24 months across four defined participant groups in the parent NP Resistome study.

Study Population:

Participants will be children aged 12–24 months enrolled in the NP Resistome study (COMREC reference P.10/24-1200) in the following groups;

- Children hospitalised with pneumonia at QECH.
- Children with pneumonia treated at primary health centres (i.e NHC).
- Healthy community controls with and without recent antibiotic exposure.

2.2. Study Period:

This sub-study is planned over an 18-month period, from July 2025 to January 2027, running alongside the NP Resistome study (Protocol v5.0 COMREC reference P.10/24-1200). Activities include sample aliquoting, international shipment, laboratory testing using the ssUAD assay, data integration, statistical analysis, and dissemination of findings.

These activities will be carried out in sequential and overlapping phases, aligned with the timelines and logistics of the parent study. Community and hospital-based participant samples collected through NP Resitome will be utilised throughout.

A detailed timeline of the sub-study activities is presented in the Gantt chart below, outlining the expected duration and sequencing of each phase.

	July -August 2025	September-October 2025	November-December 2025	January-February 2026	March – April 2026	June- July 2026	August – September 2026	October-November 2026	December 2026 – January 2027
Urine sample aliquoting and storage									
Shipping of urine samples to the UK									
ssUAD testing at Nottingham/UK HSA **Compile carriage and metagenomic data** **Data cleaning and linkage**									
Data analysis (prevalence, regression, ROC)									
Write-up and report preparation									

Dissemination (thesis, papers, conferences)									

Figure 1. Study timeline

All urine samples collected as part of this sub-study will be stored at the Malawi-Liverpool-Wellcome (MLW) Programme for up to five years following study completion, in accordance with institutional and ethical guidelines. Upon conclusion, final reports will be submitted to COMREC, the Blantyre District Health Office, and the study sponsor (LSTM), alongside dissemination to scientific and policy stakeholders.

2.3. Inclusion and Exclusion criteria:

This sub-study will use existing urine samples that have already been collected as part of the NP Resistome study (COMREC reference P.10/24-1200). The inclusion criteria applied during that study were: children aged 12–24 months residing in Blantyre District whose parents or legal guardians provided written informed consent.

As no new participant recruitment or sample collection will be undertaken, no additional inclusion or exclusion criteria are applicable for this sub-study.

2.4. Sample collection and handling:

This sub-study will use existing urine specimens that have already been collected as part of NP Resistome study. Up to 5 ml of urine was collected per participant using sterile paediatric urine collection bags. Each specimen was aliquoted into three 1.5 ml cryovials and stored at –80 °C until analysis. All specimens were Pseudonymised and linked to participants only through study ID numbers, in accordance with data protection protocols.

2.5. Laboratory analysis:

Urine samples will be analysed using the ssUAD, a Luminex-based immunoassay designed to detect pneumococcal polysaccharide capsules. Testing will follow validated protocols and will be conducted at an accredited reference laboratory with expertise in multiplex immunoassay

techniques at the University of Nottingham NHS Trust, UKHSA and PATH based in Seattle, USA.

2.6. Sample size:

The minimum required sample size for this study is 147 urine specimens, accounting for a 10% allowance for non-response or data loss. This estimate is based on detecting a significant difference in pneumococcal serotype-specific antigen detection between children hospitalised with pneumonia, those treated at health centres, and healthy community controls.

The core sample size calculation indicates that at least 49 specimens per group are needed, assuming antigen detection rates of 70% in hospitalised children, 53% in health centre cases, and 35% in healthy controls, based on prior studies[8,9]. Using a two-sided significance level of 0.05 and 80% statistical power, the estimated minimum total sample size is 147 participants across the three groups.

To address the limitation of a small sample size faced in our preliminary analysis of 214 urine specimens from hospitalised children, we plan to test all 350 urine specimens collected under the parent NP Resistome study. This expanded approach will improve the precision of our estimates, allow for subgroup analyses, and provide broader insights into pneumococcal antigen detection patterns across the study groups

2.7. Data management:

All data generated from this sub-study will be managed in alignment with the main NP Resistome study (COMREC reference P.10/24-1200). Urine specimens will be labelled using the same barcoded identifiers already assigned under the NP Resistome framework. No new study identifiers will be created.

Data will be entered into a secure, password-protected electronic database with access restricted to authorised study personnel. To ensure data accuracy and completeness, double data entry procedures and regular validation checks will be implemented.

All data will be used solely for the objectives described in this protocol and handled in accordance with institutional data governance policies and COMREC ethical approvals. No personally identifiable information will be included in shared datasets or reported results.

2.8. Data analysis:

The data analysis plan is structured to address the study's three specific objectives:

2.8.1. Prevalence and distribution (objective 1):

Descriptive statistics (frequencies, means, medians) will be used to summarise participant characteristics across study groups. Serotype-specific prevalence of urinary pneumococcal antigens will be calculated for each group, hospitalised pneumonia, community-managed pneumonia, and healthy controls. Differences between groups will be assessed using chi-square tests or Fisher's exact test, as appropriate.

2.8.2. Association with carriage dynamics (objective 2):

Logistic regression models will be employed to assess the association between urinary antigen detection (the outcome variable) and nasopharyngeal carriage (the predictor), adjusting for potential confounders such as age, antibiotic use, and HIV status. Subgroup analyses will examine the influence of carriage density and serotype co-carriage on antigen detection. Multivariable regression and interaction terms will help explore effect modification.

2.8.3. Defining diagnostic thresholds (objective 3):

Signal intensity data (median fluorescence intensity or equivalent) from the 24ssUAD assay will be analysed to compare antigen profiles between cases (pneumonia) and carriers (healthy children). Receiver Operating Characteristic (ROC) curve analysis will be used to define optimal cut-off values for distinguishing pneumococcal disease from carriage. Area under the curve (AUC) values will quantify diagnostic performance. Findings will be cross-referenced with previously published UK datasets to support broader applicability.

All analyses will be conducted using R software, with statistical significance defined at $p < 0.05$ and confidence intervals at 95%. Graphical data presentation will include bar charts, ROC curves, and stratified prevalence plots.

2.9. Data sharing:

Data generated from this sub-study will be shared with collaborators under restricted-access data sharing agreements. All shared datasets will be de-identified and used solely for the purposes outlined in this protocol, in accordance with institutional and ethical guidelines.

3.0 Quality assurance and control

All laboratory analyses will be conducted using standardised protocols at the UK Health Security Agency (UKHSA) and NIHR Nottingham Biomedical Research Centre. Internal quality control (QC) procedures and reproducibility assessments will be implemented throughout the testing process to ensure data integrity and reliability.

4.0 **Dissemination plan**

Study findings will be disseminated through multiple channels, including submission to the College of Medicine Research and Ethics Committee (COMREC), presentations at national conferences, such as the Research Dissemination Conference (RDC), and international scientific conferences, as well as publication in peer-reviewed journals. A summary of findings will also be shared with local stakeholders, including clinical and public health partners in Malawi.

5.0 Ethical consideration

The parent NP Resistome study (COMREC reference P.10/24-1200) has obtained ethical approval from all relevant institutional review boards. Written informed consent for participant enrollment will be obtained from parents or guardians. Informed consent will be obtained from the parents or guardians of participants for the testing of stored urine samples (collected as part of the NP Resistome study) using urinary pneumococcal antigen assays, with the assistance of a separate patient information leaflet and informed consent form. All procedures will be conducted in accordance with the approved ethical protocol and institutional research governance policies.

6.0 Participant compensation

Participants in this sub-study will receive no additional compensation beyond what is already provided for participation in the NP Resistome study (COMREC reference P.10/24-1200). All participants in the NP Resistome study receive 17,000 MWK as compensation, which covers all study-related procedures, including urine sample collection.

7.0 Project risks and constraints

This sub-study is embedded within the NP Resistome study (COMREC reference P.10/24-1200), which will obtain all relevant ethical approvals and is poised to begin participant recruitment. While leveraging the infrastructure and participant pool of the parent study offers significant advantages, there remain potential risks and constraints specific to the successful implementation of the urinary pneumococcal antigen sub-study.

7.1. Dependency on NP Resistome recruitment timeline

The sub-study relies on timely and sufficient recruitment within the NP Resistome study (COMREC reference P.10/24-1200) to prospectively collect urine samples from children with pneumonia and healthy controls. Any delays or slower-than-expected enrolment in the parent study could limit the number of available urine specimens. To mitigate this, the sub-study team will maintain close coordination with the NP Resistome field team to ensure that urine collection is integrated into the enrolment process for each eligible participant from the outset.

7.2. Difficulty in collecting adequate urine volumes

Obtaining sufficient urine volumes, particularly from young children, may pose practical challenges in both community and hospital settings. Missed collections due to dehydration or discomfort could affect sample availability. To mitigate this, caregivers will be supported by trained staff using child-friendly collection protocols.

7.3. Laboratory assay limitations or technical errors

The ssUAD assay, although robust, involves complex multiplex detection that requires strict standardisation and quality control to minimise risks such as batch variability, technical failures, or low signal intensity. All testing will be conducted at the NIHR Nottingham Biomedical Research Centre and UKHSA under validated SOPs, with internal quality control and pilot validation runs to ensure assay reliability prior to full sample processing. While a

Luminex platform is available locally at MLW, current capacity is limited due to a lack of validated reagents, technical expertise, and established protocols specific to ssUAD testing. Therefore, testing will be performed in UK laboratories with the necessary infrastructure and experience. Plans are underway to build local capacity and eventually introduce and evaluate the assay in Malawi as part of future implementation efforts.

7.4. Interpretation challenges due to high carriage prevalence

In a high-carriage setting like Malawi, interpreting the clinical significance of urinary pneumococcal antigen detection, especially among asymptomatic individuals, may be challenging. If urinary antigen is detected in individuals without pneumococcal carriage, or vice versa, this could complicate efforts to set reliable thresholds for distinguishing disease from carriage. To address this, the sub-study will incorporate detailed nasopharyngeal carriage data and apply ROC curve analysis to refine diagnostic interpretation.

8.0 Budget

Table 1. Estimated study budget (1 GBP = 2200 MWK)

Budget Item	Sub Item	Cost (£)	Cost (MWK)
1. Urine analysis	Testing of 350 samples at £30/sample	£10,500.00	MWK23,100,000
2. Shipping costs	International courier to Nottingham University	£2,500.00	MWK5,500,000
	Packaging and cold chain materials like dry ice	£1,500.00	MWK3,300,000
3. Laboratory consumables	Pipette tips, cryoboxes and gloves	£2,363.00	MWK5,198,600
Total		**£16,863.00**	**MWK37,098,600**

8.1. Budget justification

8.1.1. Urine Analysis:

This will support the urine analysis and optimisation of the ssUAD assay at the University of Nottingham NHS Trust and UKHSA.

8.1.2. Shipping:

This will contribute to the costs of shipping the urine samples to the UK for further analyses as outlined in the protocol.

8.1.3. Laboratory consumables:

This will support the purchase of laboratory consumables in Malawi for the preparation of urine specimens.

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