**Objectives**:

This experiment aimed to develop and validate a multiplex quantitative Polymerase Chain Reaction (qPCR) assay to screen sputum samples for three fungal species using previously published primer-probe sequences*.* In Low-and-Middle-Income Countries (LMIC) like The Gambia, detecting invasive pulmonary fungal infections can be challenging given the relative lack of site-specific diagnostic tests. This assay was developed to provide a baseline screening modality and potentially aid in early detection and initiation of therapy.

**Materials & Methods:**

The experiments were conducted at The Medical Research Council Unit The Gambia at the London School of Hygiene & Tropical Medicine and initially tested for in vitro cross-reactivity on whole DNA extract from fungal species at the University of California Los Angeles.

Primers and probes were selected to target mitochondrial small-subunit RNA (mtSSU) gene for *Histoplasma spp*., internal transcribed spacer (ITS) region for *Aspergillus spp*., and mitochondrial large-subunit ribosomal RNA (lsrRNA) gene for *Pneumocystis jirovecii*, based on published sequences with minor modifications and human ribonuclease-P (RNaseP) as an internal control. In silico validation using Primer-BLAST confirmed specificity for all primer-probe sets, with minor but clinically insignificant cross-reactivity noted between *Aspergillus* and *Penicillium* spp.. DNA was extracted using pre-treatment with lyticase followed by QIAMP® DNA Mini Kit purification.

Real-time qPCR was performed on a Bio-Rad® CFX96 system in 5μL reactions using Universal TaqMan® Master Mix, with cycling conditions of 50°C for 2 minutes, 95°C for 10 minutes, and 39 cycles of 95°C for 15 seconds and 55°C for 1 minute. Assay sensitivity and efficiency were determined through 10-fold serial dilutions of synthetic double-stranded DNA controls ranging from 10³-100 copies and analysed using linear regression standard curves. Reaction efficiencies were determined by calculating the slope of the curves. The theoretical limit of detection (LOD) was validated by testing replicate multiplex reactions near the theoretical LOD, confirming sensitivity and specificity.

**Results**:

No cross-reactivity was observed with non-target species during the in vitro specificity tests. Amplification was detected across all serial dilutions at singleplex and multiplex conditions, with acceptable Ct (cycle threshold) ranges for each target. The reaction efficiency under singleplex conditions was calculated at 95% (Histoplasma spp.), 110% (Aspergillus spp.), and 95% (Pneumocystis jirovecii), multiplex efficiencies of 80%, 99%, for *Pneumocystis jirovecii* and *Aspergillus spp,* respectively, while there was an acceptable 15% reduction for *Histoplasma spp.*.

At 5 copies per reaction, *Pneumocystis jirovecii* and *Aspergillus spp.* maintained 100% sensitivity, while for *Histoplasma spp*. sensitivity dropped to 80%. No non-specific amplification was observed in the multiplexed replicate experiments or in the in vitro specificity panel. Amplification plot analysis established a Ct threshold value of 40 for all targets.

**Conclusions**:

This study validated a multiplex qPCR assay for reliable detection of three target fungal pathogens in sputum. We found that the assay had acceptable sensitivity and specificity. Coupled with the relative ease of use and high-throughput nature of qPCR allowed us to seamlessly integrate the assay into preexisting workflows in our laboratory. Future work and clinical validation are needed to determine if the test is useful in the resource-limited setting.