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DRUG DISCOVERY AND RESISTANCE

Detection and characterization of drug-resistant conferring genes in *Mycobacterium tuberculosis* complex strains: A prospective study in two distant regions of Ghana

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SUMMARY

We spoligotyped and screened 1490 clinical *Mycobacterium tuberculosis* complex strains from Northern and Greater Accra regions of Ghana against INH and RIF using the microplate alamar blue phenotypic assay. Specific drug resistance associated genetic elements of drug resistant strains were analyzed for mutations. A total of 111 (7.5%), 10 (0.7%) and 40 (2.6%) were mono-resistant to INH, RIF, and MDR, respectively. We found the Ghana spoligotype to be associated with drug resistance (INH: 22.1%; $p = 0.0000$, RIF: 6.2%; $p = 0.0103$, MDR: 4.6%; $p = 0.0240$) as compared to the Cameroon spoligotype (INH: 6.7%, RIF: 2.4%, MDR: 1.6%). The propensity for an isolate to harbour *katG* S315T mutation was higher in *M. tuberculosis* (75.8%) than *Mycobacterium africanum* (51.7%) ($p = 0.0000$) whereas the opposite was true for *inhApro* mutations; MAF (48.3%) compared to MTBSS (26.7%) ($p = 0.0419$). We identified possible novel compensatory INH resistance mutations in *inhA* (G204D) and *ahpCpro* (-88G/A and -142G/A) and a novel *ndh* mutation K32R. We detected two possible *rpoC* mutations (G332R and V483G), which occurred independently with *rpoB* S450L, respectively. The study provides the first evidence that associate the Ghana spoligotype with DR-TB and calls for further genome analyses for proper classification of this spoligotype and to explore for fitness implications and mechanisms underlying this observation.

1. Introduction

Tuberculosis (TB) remains an important global health problem, with close to 9 million new cases per year and a pool of approximately 2 billion latently infected individuals worldwide [1]. Of particular concern are the on-going epidemics of drug resistant TB (DR-TB), which threaten to make TB incurable. The main causative agents for human tuberculosis are *Mycobacterium tuberculosis* sensu stricto (MTBSS) and *Mycobacterium africanum* (MAF), both members of the *M. tuberculosis* complex (MTBC). Of particular interest to West Africa is MAF, which causes up to 50% of human TB in some countries [2].

Drug resistance to isoniazid (INH) and rifampicin (RIF), the two backbone-drugs of the directly observed treatment short-course (DOTS) regimen, can negatively affect the successful outcome of treatment of TB [3,4]. The pro-drug INH requires activation by catalase peroxidase encoded by the *katG* of the target bacterium. Activated INH disturbs cell wall synthesis by binding to a 2-trans-

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enoyl-acyl carrier protein reductase (encoded by *inhA*) required for mycolic acid synthesis [5]. Rifampicin kills bacteria by halting the elongation of a nascent polynucleotide by occluding the path of the growing RNA through the polymerase [6]. *katG* (mostly S315T) and *inhApro* (mostly -15C/T) mutations are associated with high and low level INH resistance respectively [7]. Even though the *katG* mutation (inactive INH) and *inhApro* substitutions (overproduction of inhA) are the major causes of INH resistance, other loci such as *ndh, inhA* and *ahpCpro* have either been implicated with drug resistance or restoring the fitness cost associated with some high level resistance mutations [8,9]. Some *rpoB* mutations alter the 3D structure of the *rpoB* hence affecting the conformation of the RIF-binding pocket which in effect prevents the rigid structure of RIF from binding to the bacterial polymerase to inhibit transcription [6,10]. Even though *rpoB* mutations account for most RIF resistance in TB, mutations within the *rpoA* and *rpoC* have also been implicated with restoring the fitness cost associated with the acquisition of *rpoB* mutations [11]. Both *katG* S315T and *rpoB* S450L mutations (the most dominant INH and RIF resistance associated mutations respectively) have been associated with no/low fitness cost which may explain their dominance in clinical MTBC strains even though they confer high-level resistance [12,13].

Though non-compliance of therapeutic regimen may contribute to the development of DR-TB, the contribution of the infecting bacteria cannot be underestimated. The Beijing strain of lineage 2 is associated with hyper-virulence and drug resistance even though reports on drug resistance are sometimes conflicting [14]. On the other hand, Lineage 1 is reported to be less virulent and negatively associated with high cost mutations, which are mostly responsible for high-level drug resistance [13–15]. This indicates that different genotypes of the MTBC may be associated with resistance to specific drugs and these associations may be driven by specific genetic alterations.

In this study, we screened MTBC strains isolated from pulmonary TB patients reporting to selected health centres from the Northern and Greater Accra Regions of Ghana for drug resistance to INH and RIF. We analysed specific genetic elements for DR associated mutations by DNA sequencing of implicated genes and looked for associations among drug resistance, bacterial genotypes and origin of the isolates.

2. Methodology

2.1. Ethical statement and participant enrolment

The Institutional Review Board of the Noguchi Memorial Institute for Medical Research (NMIMR) approved the study and its protocols. Following informed consent, consecutive sputum smear-positive TB cases who were not already taken anti-TB drugs or have been put on therapy for not more than 2 weeks were recruited from all TB diagnostic centres in the Accra Metropolitan (Southern Region) and the Mamprusi East (Northern Region) Health Administrations (Figure 1) from August 2012 to September, 2014. The standard procedure for sputum sample collection as outlined by the National Tuberculosis Control Program (NTP) for routine diagnosis of TB in Ghana was followed. Informed written consent was sought from all participants unless the participant was illiterate; in which case witnessed oral consent was used. Consent was sought from guardians of children below the age of 18 before enrolment into the study and in some cases child assent was also sought. Data collected from enrolled patients included age, sex, bacterial burden on day of diagnosis and TB treatment history.

2.2. Mycobacterial isolates

The sputum samples were decontaminated with 5% oxalic acid [16], and inoculated on Lowenstein-Jensen Media (supplemented with either glycerol or pyruvate) slants, incubated at 37 °C until growth was observed. Colonies from positive cultures were sub-cultured on similar media and incubated as above until confluent growth was observed. The isolates (1490) were confirmed MTBC by PCR amplification of IS*6110*, genotyped as MTBSS, MAF and/or *Mycobacterium bovis* by large sequence polymorphism (LSPs) detecting region of difference (RD) 4, 9 and 12 [17] and spoligotyping as described by Kamerbeek et al. [18].

2.3. Anti-TB drug susceptibility testing

Micro-plate alamar blue assay (MABA) for drug susceptibility testing (DST) was performed in clear-bottomed, 96-well microplates (Nunc International, Rochester, NY, US). Drugs stocks were prepared by following standard protocols [19], and MABA-DST set up by following a modification of the method described by Franzblau et al. [20].

In summary, mycobacterial inoculum was prepared by emulsifying a loop full of logarithmic growing mycobacteria in sterile Middlebrook 7H9 [Difco, Detroit, Michigan] broth supplemented with 0.2% glycerol [Sigma-Aldrich, Steinheim, Germany] and 0.001% casitone [Difco] (7H9-GC) and adjusted spectrophotometrically to $OD_{600} = 1.0$ containing approximately 1×10^8 cfu/mL. This suspension was diluted 1:25 with 7H9-GC for inoculation. All perimeter wells of the plate were filled with 200 uL of sterile water to prevent evaporation of the content of inner wells. The test wells consisted of 7H9-GC/Tween 80 [Sigma-Aldrich] medium and respective concentration of testing drugs, (which were serially diluted to final concentrations of 0.03125–1.0 ug/mL and 0.0625 to 2.0 ug/mL for INH and RIF respectively) before inoculation with 100 μL of the mycobacterial suspension containing approximately 4×10^6 cfu/mL. The plates were incubated at 37 °C for 7 days, and bacterial viability was tested with freshly prepared 1:1 v/v of alamar blue reagent [Trek Diagnostic Systems, OH, USA] and 10% Tween 80. The minimum inhibitory concentration (MIC) defined as the concentration of drug in the first blue well for each drug, was then recorded (S1). The critical drug concentration was $\leq$0.25 ug/mL for both drugs [20]. The positive and negative controls for each set were drug-free media inoculated with bacterial suspension and un-inoculated containing media only respectively.

2.4. Isolation of genomic DNA

The protocol used for the extraction of genomic DNA was a synthesis of the protocols of Van Soolingen et al. [21] and Käser et al. [22]. Briefly, the mycobacterial cell wall was disrupted by adding lysozyme (50 μL lysozyme of 10 mg/mL) vortexed and incubated overnight, followed by addition of 75 μL of 10% SDS, 10 μL proteinase K (20 mg/mL), vortexed softly and incubated 15 min at 65 °C. After, the incubation, 100 μL of 5 M NaCl was added, followed by 100 μL CTAB which was pre-warmed at 65 °C. After vortexing, the extracted DNA was purified by chloroform/isoamyl alcohol extraction. The DNA contained in the upper phase was precipitated with isopropanol and washed with ethanol. The dried DNA was then re-suspended in 100 uL Tris EDTA buffer and stored at 4 °C until use.

2.5. PCR amplification and DNA sequencing of genetic elements

Drug resistant isolates were used for targeted sequencing analyses. Eight drug resistance associated genetic elements (*rpoB*, *rpoA*