In Silico Prediction of Antibiotic Resistance in *Mycobacterium ulcerans* Agy99 through Whole Genome Sequence Analysis

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Abstract. Buruli ulcer is an emerging infectious disease caused by *Mycobacterium ulcerans* that has been reported from 33 countries. Antimicrobial agents either alone or in combination with surgery have been proved to be clinically relevant and therapeutic strategies have been deduced mainly from the empirical experience. The genome sequences of *M. ulcerans* strain AGY99, *M. ulcerans* ecovar liflandii, and three *Mycobacterium marinum* strains were analyzed to predict resistance in these bacteria. Fourteen putative antibiotic resistance genes from different antibiotics classes were predicted in *M. ulcerans* and mutation in *katG* (R431G) and *pncA* (T47A, V125I) genes were detected, that confer resistance to isoniazid and pyrazinamide, respectively. No mutations were detected in *rpoB*, *gyrA*, *gyrB*, *rpsL*, *rrs*, *emb*, *ethA*, 23S ribosomal RNA genes and promoter region of *inhA* and *ahpC* genes associated with resistance. Our results reemphasize the usefulness of in silico analysis for the prediction of antibiotic resistance in fastidious bacteria.

BACKGROUND

Mycobacterium ulcerans (MU) is the causative agent of Buruli ulcer (BU) in immunocompetent hosts, which ranks third among human mycobacteriosis worldwide after tuberculosis and leprosy.¹ This has been reported from 33 countries with tropical, subtropical, as well as temperate climates.² The mode of transmission of this extracellular pathogen is still not clear. However, living agents such as aquatic insects, adult mosquitoes, or other biting arthropods may facilitate transmission.³ Until 2004, surgical debridement with or without subsequent skin grafting was the only therapeutic option, though recurrences were frequently observed.⁴ The balance between surgery and antibiotics use was shifted when mouse foot-pad model was used for antibiotic susceptibility testing (single dose rifampin and sometimes fluoroquinolones were administered in addition to surgical intervention).^{5–7} Although monotherapy (strains were isolated from mice after experimental chemotherapy) was effective for certain period but consistent use of antibiotics develop resistance in the microbes.⁸ To prevent the emergence of resistant strains, combination of antibiotics was used for the treatment. In case of BU, World Health Organization recommended the oral administration of rifampin (10 mg/kg) and intramuscular streptomycin (15 mg/kg) for 8 weeks to reduce the bacterial load.^{9,10} Recently, the use of rifampin and streptomycin for 2 weeks followed by rifampin and clarithromycin for the next 6 weeks was effective in the treatment of BU.¹¹ The slow growth rate and cultural difficulties of MU¹² make this bacterium less favorable for antibiotic susceptibility testing assay. MU ecovar liflandii strain M04–2878, a MU-like *Mycobacterium* isolated from pipid frog *Silurana tropicalis*, in a European research laboratory, was reported to be resistant to isoniazid, ethambutol, rifampin, clarithromycin, and ethionamide.¹³ Recently, a new recombinant bioluminescent assay was successfully applied to evaluate the antibacterial activity

in vitro against MU,¹⁴ which showed that MU was susceptible to rifampin, streptomycin, and clarithromycin and was resistant to tetracycline, isoniazid, and erythromycin.¹⁴ Moreover, it was demonstrated that reserpine, an inhibitor of adenosine triphosphate (ATP)-binding cassette (ABC) transporter, partially restores the susceptibility of MU to tetracycline and erythromycin due to an efflux pump (a putative macrolide ABC transporter) and might be responsible for intrinsic resistance.¹⁴

One alternative to in vitro antibiotic susceptibility testing in fastidious bacteria is to predict antibiotic resistance (AR) determinants by whole genome sequence (WGS) in silico analysis as previously reported for bacteria.¹⁵ In an effort to guide further therapeutic options, we embarked into MU genome survey to find out genes and mutations potentially associated with AR. The MU Agy99 was isolated in 1999 from an ulcerative lesion on the right elbow of a female patient from the Ga District of Ghana. The genome of MU Agy99 (CP000325) is 5,631,606-base pair long and comprises one main circular chromosome with 64.5% GC content and a 174-kilobase plasmid.¹⁶ This virulence plasmid pMUM001 was transferred to MU during its divergence from *Mycobacterium marinum*.¹⁷ ARG-ANNOT database¹⁸ and RAST annotation¹⁹ were used to find existing and putative new and/or emerging AR genes and point mutations in target genes that could be associated with AR in the genome sequence of MU Agy99 as well as in MU ecovar liflandii and *M. marinum* strains genomes.

METHODS

The genome sequence of MU Agy99 was retrieved from the NCBI GenBank (CP000325). ARG-ANNOT database¹⁸ was used to predict acquired antibiotic resistance genes using BioEdit interface²⁰ and most of additional resistance gene were picked from RAST annotation.¹⁹ NCBI BLAST was performed to validate the results obtained from the local blast. Mutational analysis was done by comparing the gene sequences with reported mutational genes.^{21–27} The AR genes or mutations detected in MU Agy99 were used as a reference to analyze the presence of these AR genes or mutations in closely related genomes which included

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TABLE 1
In silico analysis of chromosomal antibiotic resistance genes and mutations associated with resistance in *Mycobacterium ulcerans* AGY99 and comparative analysis within the *Mycobacterium marinum* complex

Antibiotics class	Size (aa)	Function	Best Hit NCBI	Putative ARG	% Identity			
					<i>Mycobacterium liflandii</i>	<i>Mycobacterium marinum</i> M	<i>Mycobacterium marinum</i> Europe	<i>M. marinum</i> MB2
Aminoglycoside	276	Aminoglycoside 3-phosphotransferase/protein blocks polypeptide synthesis by inhibiting the elongation step	<i>M. liflandii</i> 128FXT (5549831_5550562)	aph	99.73	99.05	99.18	99.32
	181	Coenzyme A-dependent acetylation of the 2' hydroxyl or amino group	<i>M. liflandii</i> 128F (1272229_1271684)	aac2-lc	99.82	99.63	99.45	99.82
β-Lactamases	414	β-Lactamase class C and other penicillin binding proteins	<i>M. avium</i> 104 (4889836_4891941)	amp	99.43	98.38	98.43	99.10
	250	Metallo-β-lactamase superfamily protein	<i>Mycobacterium tuberculosis</i> CDC1551 (3143802_3144554)	aim	99.87	99.60	98.41	99.34
	297	β-Lactamase A	<i>M. marinum</i> M (2568033_2567140)	far	98.99	99.33	98.55	99.33
	359	β-Lactamase/D-alanine carboxypeptidase (transpeptidase superfamily)	<i>M. liflandii</i> 128FXT (2113703_2114785)	och	99.32	98.64	97.27	98.77
	379	β-Lactamase class C and other penicillin binding proteins	<i>M. marinum</i> M (1958947_1957808)	amp	99.65	99.65	99.47	99.21
	225	Predicted Zn-dependent hydrolase of the β-Lactamase fold	<i>M. liflandii</i> 128FXT (1760783_1760106)	gob	99.41	98.38	98.82	98.67
MLS/TET	272	β-Lactamase class C and other penicillin binding proteins	<i>M. liflandii</i> 128FXT (1391326_1392144)	amp	99.51	99.27	99.15	99.51
	643	Macrolide ABC transporter ATP-binding protein	<i>Mycobacterium canettii</i> C1PT 140070017 (1825164_1823233)	ery /tet	99.53	99.28	98.19	99.48
Sulfonamide	274	Dihydropteroate synthase	<i>M. liflandii</i> 128FXT (1006149_1006973)	sul	99.52	99.27	99.03	99.27
	275	Dihydropteroate synthase	<i>M. marinum</i> M (4661918_4661091)	sul	99.28	99.40	97.95	99.15
Trimethoprim	172	Disrupts DNA, RNA, and protein synthesis	<i>M. liflandii</i> 128FXT (2431765_2432253)	dfr	99.45	99.64	97.27	99.61
	182	Disrupts DNA, RNA, and protein synthesis	<i>Sanguibacter keddeii</i> DSM 10542 (3230564_3231112)	dfr	99.80	100	99.39	100
Isoniazid	708	Inhibits the synthesis of mycolic acids, an essential component of the bacterial cell wall	<i>M. liflandii</i> 128FXT (2441410_2443536)	katG Arg-431-Leu* (pseudogene)	katG Arg-431-Leu (pseudogene)	katG Arg-431-Leu	katG Arg-431-Leu	katG Arg-431-Leu
Pyrazinamide	196	Disrupted membrane energetics and inhibits membrane transport function at acidic pH	<i>M. liflandii</i> 128FXT (2510652-2511212)	pncA Thr-47-Ala Val-125-Ile†	pncA Thr-47-Ala Val-125-Ile	pncA Thr-47-Ala Val-125-Ile	pncA Thr-47-Ala Val-125-Ile	pncA Thr-47-Ala Val-125-Ile

ABC = adenosine triphosphate-binding cassette; ARG = antibiotic resistance gene (not the same gene but closely matched gene).

* Codon 431 in *M. ulcerans* corresponds to codon 463 of *M. tuberculosis* for katG gene where amino acid change at position Arg463Leu leads to INH resistance.²⁵

† The detected codon at position 125 was isoleucine in *M. ulcerans*, however resistance strains of *M. tuberculosis* had phenyl alanine in place of valine at 125, no codon changes (Ile-6-The, His-51-Gln, Pro-54-Ser, Phe-58-Leu, Gly-132-Ile etc.)²⁶ were noticed at other regions of pncA gene in MU. Hence, the amino acid change at position 125, predicted as potential mutation for resistance.

three genomes of *M. marinum* (M; NC_010612, Europe; ANPL01000001-4 and MB2; ANPM01000001-3) and MU ecovar liflandii; NC_020133). The Sequence Read Archive data (Illumina pair end reads) for 30 MU genomes (list provided as Supplemental Figure 1 and Supplemental Tables 1 and 2) were retrieved from the NCBI (<http://www.ncbi.nlm.nih.gov/sra/website>). The reads were assembled using CLC workbench7 (<http://www.clcbio.com/website>). A local database for all the genes conferring resistance (*rpoB*, *gyrA*, *gyrB*, *rpsL*, *rrs*, *pncA*, *embB*, *inhA*, *katG*, *oxyR*, *ahpC*, *ndh*, and 23S ribosomal RNA [rRNA])^{21–29} was created in BioEdit.²⁰ The assembled contigs from SRA data of MU genomes were analyzed through blast against the database and blast output was assessed for possible reported mutations.^{21–29}

Phylogenetic trees based on MU *rpoB* gene sequences were inferred using MEGA.³⁰ The *rpoB* sequences were extracted from the assembled contigs of SRA data and other genomes under study, multiple sequence alignments were carried out using CLUSTALW and phylogenetic inferences obtained using the neighbor-joining method of MEGA. The bootstrap values of consensus tree were inferred from 500 replicates. The evolutionary distances were computed using the Kimura2 parameter.³¹ All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option).

RESULTS AND DISCUSSIONS

WGS analysis of MU Agy99 revealed 14 chromosomal AR genes and mutations in *pncA* and *katG* genes (Table 1 and Supplemental Table 1). These findings were compared with the genomes of *M. marinum* (M, Europe and MB2), MU ecovar liflandii and 30 assembled genomes from SRA data (ERR449672) and analysis is presented in Table 1 and Supplemental Tables 1 and 2. Mutations were detected only in *katG* and *pncA* genes in 29/30 assembled MU genomes (Supplemental Table 2). Phylogenetic position of all the analyzed genomes is presented in Figure 1.³⁰ Interestingly all AR genes detected in MU AGY99 were also present in the four other genomes under analysis with more than 98% sequence similarity (Table 1). MU encodes an Amber class B metallo-β-lactamase (MBL), an Amber class C β-lactamase and a class A β-lactamase that may likely contribute resistance to β-lactams and resistance to β-lactams in *Mycobacterium tuberculosis* (BlaC), *Mycobacterium smegmatis* (BlaS), and *Rickettsia felis* have been described previously.^{32,33} Further, we found two aminoglycoside modifying enzymes (AMEs) encoding genes (*aac*[2']-Ic and *aph*) that are known to confer resistance to gentamicin, sisomicin, and fortimicin.³⁴ However, no genes (*ant*[3']-Ia, *aadA*, and *aad*[3']-9) or mutations in rRNA genes (*rpsL*, *rrs*, and *rpsD*) associated with resistance

FIGURE 1. Phylogenetic tree based on *rpoB* gene highlighting the position of *Mycobacterium ulcerans* (Mu) genomes within the closely related genus *Mycobacterium liflandii* (Ml), *Mycobacterium marinum* (Mm), and others. Sequences were aligned using CLUSTALW and phylogenetic inferences obtained using the neighbor-joining method within MEGA program. Numbers at the nodes are bootstrap values obtained by repeating 500 times the analysis to generate a majority consensus tree. The scale bar represents a 1% nucleotide sequence divergence.

to streptomycin were found in any of the genome analyzed here (Table 1); and *gidB* gene (streptomycin resistance) was not detected either. In silico analysis of the 23S rRNA and ribosomal proteins did not show mutations associated with resistance to macrolide compounds in other bacteria.¹⁵ However, MU genome encodes a macrolide ABC transporter ATP-binding protein (Table 1 and Supplemental Table 1) exhibiting > 75% nucleotide sequence similarity with the homologous gene in *M. tuberculosis* H37Rv which may confer resistance to erythromycin/tetracycline.^{14,35} In *M. marinum* complex, this protein is highly conserved (100%) and grouped in single clade in most of the assembled genomes except Mu_SRX031535 genome (Supplemental Figure 1). No other gene was found to confer intrinsic resistance to macrolides.^{33,36} The MU genome contains two copies of dihydropteroate synthase and dihydrofolate reductase that may account for possible resistance to sulfonamide and trimethoprim. This needs to be explored further to clarify whether this bacterium is resistant to such compounds.³⁷ Interestingly, an Arg431Leu mutation in *katG*, known to confer isoniazid resistance corresponding to position 463 in *M. tuberculosis* was found in all studied genomes.^{25,26} Though, Ser315Gly in *katG* is the most frequent mutation reported in *M. tuberculosis*,²³ we also detected serine in *katG* gene at position 284 in the alignment for MU, which corresponds to position 315 of *M. tuberculosis*. The analyzed *pncA* gene showed amino acid change at position T47A (except all three *M. marinum*, *Mycobacterium liflandii*, and Mu_SRX031535) and V125I in all analyzed genomes, which confer resistance to pyrazinamide in *M. tuberculosis*. Moreover, we analyzed all 18 reported mutations in *pncA* gene in *M. tuberculosis* and noticed amino acid change at two positions (T47A and V125I) in MU, though the amino acid change at position 125 was V125I in MU as compared with V125F in *M. tuberculosis*, thus, further experimental validation would be needed.²⁷ No mutations were detected in *inhA*, *katG*, *oxyR*, *ahpC*, *ndh* genes and in the promoter region of *inhA* (C15T) and *ahpC* (G32A) associated with isoniazid resistance.²⁹ No mutations were detected in *embB* and *ethA* genes to confer resistance to ethambutol and ethanamide respectively.²⁸ Finally we did not find any mutation in the rifampin resistance-determining region of *rpoB*.^{29,38} Likewise, the quinolone resistance-determining region of the *gyrA* and *gyrB* genes revealed no mutations associated with fluoroquinolone resistance.^{21,22,39}

CONCLUSIONS

Even if our analysis remains only predictive by in silico analysis such approach was successfully used in our experience for other fastidious and/or intracellular bacteria to shift from empirical to rationale antimicrobial therapy for *Bartonella*³⁹ and *Tropheryma whippelii* infections.⁴⁰ Here, we predict that isoniazid and pyrazinamide are probably ineffective agents against MU complex mycobacteria, whereas rifampin, streptomycin, azithromycin, clarithromycin, and fluoroquinolones currently used for the empirical antibiotic treatment of BU, were predicted to be effective. Very few studies has been conducted on drug resistance MU from humans.^{7,41} As acquired resistance to these antibiotics might occur by single mutation in target genes during monotherapy, antibiotics combination could be the better alternative to treat BU patients. Finally, we believe that surveillance of a possible

emergence of resistance to these antibiotics could be implemented by development of molecular tests easily monitoring any changes in these target genes.

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