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Combination of amikacin and doxycycline against multidrug-resistant and extensively drug-resistant tuberculosis

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ABSTRACT

The objective of this study was to assess the activity of amikacin in combination with doxycycline against clinical strains of *Mycobacterium tuberculosis* in the search for new strategies against multidrug-resistant (MDR) and extensively drug-resistant (XDR) tuberculosis. The study included 28 clinical *M. tuberculosis* strains, comprising 5 fully susceptible, 1 isoniazid-resistant, 17 MDR, 1 poly-resistant (streptomycin/isoniazid), 1 rifampicin-resistant and 3 XDR isolates, as well as the laboratory strain *M. tuberculosis* H37Rv. Minimum inhibitory concentrations (MICs) were determined using a modified chequerboard methodology in a BACTEC™ MGIT™ 960 System. Fractional inhibitory concentration indices (FICIs) were calculated, and synergy, indifference or antagonism was assessed. Whole-genome sequencing was performed to investigate the genetic basis of synergy, indifference or antagonism. The MIC_{50} and MIC_{90} values (MICs that inhibit 50% and 90% of the isolates, respectively) were, respectively, 0.5 mg/L and 1.0 mg/L for amikacin and 8 mg/L and 16 mg/L for doxycycline. The combination of amikacin and doxycycline showed a synergistic effect in 18 of the 29 strains tested and indifference in 11 strains. Antagonism was not observed. A streptomycin resistance mutation (K43R) was associated with indifference. In conclusion, the benefit of addition of doxycycline to an amikacin-containing regimen should be explored since in vitro results in this study indicate either synergy or indifference. Moreover, doxycycline also has immunomodulatory effects.

1. Introduction

The growing problem of multidrug-resistant tuberculosis (MDR-TB) and extensively drug-resistant tuberculosis (XDR-TB) poses a serious problem for TB control [1]. Whilst new drugs are being introduced [2,3], resistance mechanisms have already been characterised, thus new strategies are needed to preserve these drugs by avoiding the emergence of resistance and potentiating their activity by combining them with other drugs. Further strategies may include re-evaluating old drugs against *Mycobacterium tuberculosis*, such as tetracyclines, either alone or in combination to produce synergistic activity [4–6].

Doxycycline is a broad-spectrum antibiotic belonging to the tetracycline group that has been in use for almost 50 years and whose side effects are widely known. It is primarily bacteriostatic and is thought to exert its antimicrobial effect by inhibiting protein synthesis. It prevents the binding of aminoacyl-tRNA to the mRNA–30S ribosomal subunit complex, preventing the addition of new amino acids into the growing peptide chain [7].

Doxycycline has been reported to have activity against *M. tuberculosis* in the past, although 7.4% of the strains in one series tested resistant [8]. One resistance mechanism has been identified as efflux mediated by efflux pumps [9,10].

Amikacin, one of the main second-line antituberculous drugs, is an injectable drug belonging to the aminoglycoside family. It binds reversibly to ribosomes, resulting in a measurable decrease in protein synthesis as a result of misreading of mRNA [11].

Although both drugs act upon the same metabolic pathway, namely protein synthesis, by binding to the 30S subunit of the

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ribosome affecting elongation of the protein, tetracyclines inhibit tRNA delivery whilst aminoglycosides affect translocation of amino acids [11]. The synergistic effect of two antibiotics acting on two different steps in the same metabolic pathway has been used for other antibiotics such as trimethoprim/sulfamethoxazole [12]. However, it had never been tried for a tetracycline and an aminoglycoside against *M. tuberculosis*.

The purpose of this work was to assess the activity of amikacin, one of the most frequently used second-line drugs in the UK, in combination with doxycycline against clinical strains of *M. tuberculosis*.

2. Methods

2.1. Pilot study

A panel of 10 clinical strains were set up alongside a series of 10 bacteria-free tubes containing medium only (modified Middlebrook 7H9; Becton Dickinson, Franklin Lakes, NJ), oleic acid–albumin–dextrose–catalase (OADC) (Becton Dickinson) and doxycycline to assess whether there was any interference with the normal functioning of the BACTEC™ MGIT™ (Mycobacterium Growth Indicator Tube) 960 System (Becton Dickinson) in the presence of doxycycline.

2.2. Main study

2.2.1. Bacteria

In addition to the laboratory strain *M. tuberculosis* H37Rv, 28 clinical strains of *M. tuberculosis* were included in this study, comprising 5 fully susceptible, 1 isoniazid-resistant, 17 MDR, 1 poly-resistant (streptomycin/isoniazid), 1 rifampicin-resistant and 3 XDR isolates. A full susceptibility profile for all the strains is shown in Table 1.

2.2.2. Drug solutions

Stock solutions were prepared following the manufacturer's instructions, divided into 500 μL aliquots and frozen at −80 °C.

Amikacin disulphate (Sigma-Aldrich, Gillingham, UK) was dissolved in water at concentrations of 0.0625–8 mg/L.

Doxycycline hyclate (Sigma-Aldrich) was dissolved in dimethyl sulphoxide (DMSO) at concentrations of 1–64 mg/L.

Doxycycline concentrations chosen were guided by previously published human pharmacokinetic data, whilst amikacin concentrations were guided by the current critical concentration recommended by the World Health Organization (WHO) of 1 mg/L [13–18].

2.3. Drug susceptibility testing

Minimum inhibitory concentrations (MICs) for both drugs were determined using a modified chequerboard methodology using the BACTEC™ MGIT™ 960 System, adapting the methodology previously described for second-line drug susceptibility testing [19].

Following primary isolation in the BACTEC™ MGIT™ 960 System, MGIT tubes were supplemented with 0.8 mL of OADC. Then, 0.1 mL of each drug concentration was added to each tube. Finally, 0.5 mL of the culture was added. The growth control tube was prepared by diluting the original culture in saline in a 1:100 ratio. Then, 0.5 mL of this suspension was inoculated in the drug-free tube. Results were analysed using EpiCenter TB eXist software (Becton Dickinson) [20].

A repeat test was performed for every strain, generating a second set of data.

Table 1
Full susceptibility profile of tested strains against first- and second-line antituberculous agents.[a]

Strain	Susceptibility	INH	RIF	PZA	EMB	STR	AMK	CAP	MOX	OFL	ETH	PTH	KAN	LIN
H111040027	XDR	R	R	R	I	R	S	I	R	R	S	I	R	S
H37Rv	FS													
H111540004	MDR	R	R	R	S	R	S	S	S	S	NT	S	R	NT
H111500010	INH-R	R	R	R	S	R	S	S	S	S	NT	S	R	NT
H111620021	MDR	R	R	R	R	R	R	R	S	S	S	R	R	S
H111620002	MDR	R	R	S	S	S	S	S	S	S	NT	S	S	NT
H111760052	MDR	R	R	NT	NT	NT	S	S	S	S	S	S	S	NT
H111860011	MDR	R	R	S	S	S	S	S	S	NT	NT	S	S	NT
H111740353	MDR	R	R	NT	NT	NT	R	R	S	NT	S	S	R	S
H112040042	MDR	R	R	NT	NT	NT	R	R	S	S	NT	S	R	NT
H111840003	RIF-R	S	R	S	S	S	S	S	S	S	NT	S	S	NT
H111980010	POLYR	R	S	S	S	R	S	S	S	S	S	S	S	S
H111900039	FS	S	S	S	S	S	NT	NT	NT	NT	NT	NT	NT	NT
H111900041	FS	S	S	S	S	S	NT	NT	NT	NT	NT	NT	NT	NT
H112080018	FS	S	S	S	S	S	NT	NT	NT	NT	NT	NT	NT	NT
H112240073	MDR	S	S	S	S	S	NT	NT	NT	NT	NT	NT	NT	NT
H112080012	FS	S	S	S	S	S	NT	NT	NT	NT	NT	NT	NT	NT
H111880072	XDR	R	R	S	R	R	R	S	R	R	NT	R	R	S
H110860461	FS	S	S	S	S	S	S	S	S	S	NT	S	S	NT
H112140033	MDR	R	R	S	R	S	S	S	R	R	NT	S	S	NT
H112160033	MDR	R	R	S	R	S	S	S	R	R	NT	R	S	NT
H121920020	MDR	R	R	S	S	S	S	S	S	S	S	S	S	NT
H122020037	MDR	R	R	S	S	R	S	S	S	S	NT	R	S	NT
H121320018	MDR	R	R	S	S	S	S	S	R	R	NT	S	S	NT
H121860041	MDR	R	R		R		R	S	S	S	NT	S	R	S
H121160050	MDR	R	R	S	R	S	S	S	S	S	NT	S	S	S
H120640724	MDR	R	R	S	S	S	S	S	S	S	NT	R	S	NT
H121400003	XDR	R	R	R	R	R	S	S	R	R	NT	R	R	S
H121460028	MDR	R	R	S	S	S	S	S	S	S	S	S	S	S

INH, isoniazid; RIF, rifampicin; PZA, pyrazinamide; EMB, ethambutol; STR, streptomycin; AMK, amikacin; CAP, capreomycin; MOX, moxifloxacin; OFL, ofloxacin; ETH, ethionamide; PTH, prothionamide; KAN, kanamycin; LIN, linezolid; XDR, extensively drug-resistant; FS, fully susceptible; MDR, multidrug-resistant; INH-R, isoniazid-resistant; RIF-R, rifampicin-resistant; POLY, poly-resistant; R, resistant; I, intermediate; S, susceptible; NT, not tested.

[a] These results were obtained prior to this study as part of Public Health England National Mycobacterium Reference Laboratory's diagnostic services, by resistance ratio (INH, RIF, EMB and STR), biphasic method (PZA) and BACTEC™ MGIT™ 960 System (AMK, CAP, KAN, LIN, ETH, PTH, MOX and OFL).

Table 2
Minimum inhibitory concentrations (MICs) and fractional inhibitory concentration indices (FICIs) for the tested strains.

Strain	First run				Repeat testing			
	MIC DOX	MIC AMK	FICI	Interpretation	MIC DOX	MIC AMK	FICI	Interpretation
H111040027	16	0.5	0.62	Indifference	16	0.5	0.75	Indifference
H37Rv	16	0.25	0.625	Indifference	16	0.5	0.75	Indifference
H111540004	8	0.5	0.374	Synergy	16	0.5	0.75	Indifference
H111500010	16	0.5	0.249	Synergy	16	0.5	0.625	Indifference
H111620021	8	16	2	Indifference	8	16	2	Indifference
H111620002	8	0.25	0.75	Indifference	8	0.25	1	Indifference
H111760052	16	0.5	0.249	Synergy	16	0.5	0.25	Synergy
H111860011	8	0.25	0.245	Synergy	8	0.25	0.25	Synergy
H111740353	8	2	0.375	Synergy	8	2	0.625	Indifference
H112040042	16	0.25	0.2498	Synergy	16	0.25	0.25	Synergy
H111840003	4	0.25	2	Indifference	8	0.25	2	Indifference
H111980010	16	0.25	0.245	Synergy	16	0.25	0.25	Synergy
H111900039	16	0.25	0.2498	Synergy	16	0.5	0.1868	Synergy
H111900041	8	0.5	0.249	Synergy	16	0.5	0.1865	Synergy
H112080018	2	0.5	0.625	Indifference	2	0.5	0.75	Indifference
H112240073	16	0.25	1	Indifference	16	0.25	2	Indifference
H112080012	16	0.5	0.625	Indifference	16	0.5	0.75	Indifference
H111880072	8	1	0.75	Indifference	8	1	1	Indifference
H110860461	8	0.5	0.375	Synergy	8	0.5	0.75	Indifference
H112140033	8	0.25	0.245	Synergy	8	0.25	0.38	Synergy
H112160033	8	0.125	0.746	Indifference	8	0.25	0.75	Indifference
H121920020	16	0.5	0.245	Synergy	16	0.5	0.25	Synergy
H122020037	8	0.5	0.249	Synergy	8	0.5	0.38	Synergy
H121320018	8	0.5	0.245	Synergy	8	0.5	0.375	Synergy
H121860041	16	1	0.25	Synergy	16	1	0.38	Synergy
H121160050	16	0.25	0.37	Synergy	16	0.25	0.62	Indifference
H120640724	16	0.25	0.245	Synergy	16	0.25	0.25	Synergy
H121400003	8	0.5	1	Indifference	16	0.5	0.75	Indifference
H121460028	8	0.125	0.245	Synergy	8	0.125	0.245	Synergy

DOX, doxycycline; AMK, amikacin.

2.4. Fractional inhibitory concentration index analysis and interpretation

Fractional inhibitory concentrations (FICs) were calculated as follows:

FIC of amikacin (FICa) = MIC of amikacin in combination/MIC of amikacin alone
FIC of doxycycline (FICb) = MIC of doxycycline in combination/MIC of doxycycline alone

The fractional inhibitory concentration index (FICI) of the two compounds in the combination was calculated as follows: FICI = FICa + FICb.

FICIs were interpreted as follows: <0.5 synergy; 0.5–4 indifference; and >4 antagonism [21,22].

2.5. Whole-genome sequencing analysis

Genomic DNA was sequenced on an Illumina MiSeq platform (Illumina, San Diego, CA) at the Queen Mary College Genome Centre (London, UK). Reads were mapped to the H37Rv reference genome using SMALT (http://www.sanger.ac.uk/resources/software/smalt). High-quality single nucleotide polymorphisms (SNPs) were identified and variant positions were used to construct a maximum likelihood tree as previously described [23]. Homoplasies were considered valid if the site passed quality filters in >90% of isolates.

Statistical analysis was performed using an online tool available at http://vassarstats.net/odds2x2.htlm.

3. Results

The pilot study indicated that the reagents had no effect on the detection/sensor system of the BACTEC™ MGIT™ 960 System.

Table 3
Mutations conferring resistance to first- and second-line drugs detected by whole-genome sequencing.

Strain	Gene											
	katG	P_*inhA*	*rpoB*	*pncA*	P_*embA*	*embB*	*rpsL*	*rrs*	P_*eis*	*gyrA*	P_*ethA*	*ethA*
H111740353	S315T	.	S450L	K96R	.	M306V	K43R	c517t	g-14a	.	.	Q165P
H111880072	S315T	.	S450L	P62S	.	M306V	K43R	c517t	g-14a	D94A	.	.
H121860041	S315T	.	D435V	P54A	.	M306I	.	c517t	g-14a	.	.	.
H111540004	S315T	.	S450L	Q141P	.	D354A	.	c517t	c-37a	.	a-7g	.
H111620021	S315T	.	S450L	W68G	.	M306V	K43R	a1401g	.	.	.	.
H112240073	S315T	.	S450L	L4S	.	Q497R	K43R	.	.	.	.	.
H111040027	S315T	.	H445Y	D12N	c-8t	.	K43R	.	c-10t	D89G	.	.
H121160050	S315T	.	S450L	.	.	.	.	.	.	.	.	.
H121320018	S315T	.	S450L	.	.	.	.	.	.	.	.	H22R
H111980010	S315T	.	.	.	.	M306I	.	.	.	.	.	.
H112160033	S315T	c-15t	S450L	L120R	c-12t	M306Q	.	.	.	D94G	.	M409V
H111860011	S315T	.	S450L	.	.	.	.	.	.	.	.	.
H112040042	.	.	.	.	.	.	.	.	.	.	.	.
H111900039	.	.	.	.	.	.	.	.	.	.	.	.
H111620002	S315T	.	D435Y	.	.	M306I	.	.	.	.	.	.
H112140033	S315T	.	L430P;D435G	.	.	M306V	.	.	.	D94G	.	.
H111840003	.	.	H445Y	.	.	.	.	.	.	.	.	.
H111500010	.	c-15t	.	.	.	.	.	.	.	.	.	.
H121920020	.	c-15t	.	.	.	.	.	.	.	.	.	.
H112080012	.	c-15t	.	.	.	.	.	.	.	.	.	.
H110860461	.	.	.	.	.	.	.	.	.	.	.	.
H111900041	.	.	.	.	.	.	.	.	.	.	.	.
H122020037	S315T	.	S450L	.	.	.	.	.	.	.	.	M1R
H120640724	.	c-15t	H445Y	.	.	.	.	.	.	.	.	G71R
H112080018	.	.	.	.	.	.	.	.	.	.	.	.
H111760052	S315T	.	S450L	.	.	M306V	K88T	.	.	.	.	N345K

In the main study, the MIC_{50} (MIC that inhibits 50% of the isolates) was 0.5 mg/L for amikacin and 8 mg/L for doxycycline. The MIC_{90} (MIC that inhibits 90% of the isolates) was 1 mg/L for amikacin and 16 mg/L for doxycycline. On repeat, the MIC_{50} for doxycycline was 16 mg/L, with no changes for the other parameters.

The combination of amikacin and doxycycline showed a synergistic effect in 18 of the 29 strains tested and indifference (additive effect) in 11 strains. On repeat testing, synergy was observed in 13 strains and indifference in 16. Antagonism was not observed. The complete set of MICs and FICIs is shown in Table 2.

When stratifying by susceptibility pattern, three of the six fully susceptible strains showed synergy [odds ratio (OR) = 1.875, 95% confidence interval (CI) 0.3051–11.5245; $P = 0.646$] and 13 of the 17 MDR strains showed synergy (OR = 4.55, 95% CI 0.9149–22.6281; $P = 0.119$). The three XDR strains showed indifference (OR = 0.1184, 95% CI 0.0115–1.2177; $P = 0.066$). Of the other three strains with unique patterns of susceptibility, two showed synergy and one showed indifference. No susceptibility pattern was statistically associated with synergistic or indifferent effect of amikacin/doxycycline (Table 2).

On repeat testing, two fully susceptible strains showed synergy (OR = 0.5455, 95% CI 0.0829–3.59; $P = 0.6628$) and ten of the MDR strains showed synergy (OR = 4.2857, 95% CI 0.844–21.7627; $P = 0.12974$). All three XDR strains continued to show indifference. Of the other three strains with unique patterns of susceptibility, two showed indifference and one showed synergy. The mutations conferring resistance to first- and second-line drugs detected by whole-genome sequencing can be seen in Table 3. None of these mutations were associated with the synergistic phenotype.

Whole-genome sequencing analysis showed that the synergistic phenotype was not concordant with any particular lineage or clade. Homoplasies, that is SNPs that have independently arisen multiple times by convergent evolution, were identified at 31 sites (Table 4; Supplementary Table S1). A SNP in the ribosomal protein gene *rpsL* (Rv0682) was significantly associated with the indifferent phenotype (OR = 12, 95% CI 1.0737–134.1161; $P = 0.040$). This SNP encodes the substitution K43R, which confers resistance to streptomycin. Isolate H111620021, which exhibited a high MIC for amikacin, had a SNP at position 1401 in the 16S rRNA gene *rrs* (MTB000019) that is associated with resistance to this drug [24].

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijantimicag.2014.11.017.

4. Discussion

Doxycycline alone was active against a few strains only; 2 were susceptible, 14 yielded intermediate susceptibility and 13 were resistant based on Clinical and Laboratory Standards Institute (CLSI) cut-offs for Gram positive micro-organisms [25].

Doxycycline has been successfully used in vitro against *M. tuberculosis* [8]. A related drug, minocycline, was used to successfully treat a patient with XDR-TB and has been tested for synergy with isoniazid plus rifampicin [5] and the fluoroquinolone lomefloxacin [26]. Older members of the tetracycline family (tetracycline, oxytetracycline and chlortetracycline) showed activity against *M. tuberculosis* strains in animal models [27,28].

More recently, doxycycline has been associated with inhibition of mycobacterial growth via inhibition of matrix metalloproteinase secretion from human macrophages, an immunomodulatory effect [29], in addition to its antibacterial effect.

In this study, we have found that there is either an indifferent or synergistic effect between amikacin and doxycycline, adding an additional advantage to using it as part of a second-line anti-TB treatment. In previous reports with older members of the tetracycline family in combination with streptomycin or isoniazid, the same was found [30]. As in that study, the presence of synergy or indifference was not systematic or predictable and was not associated with any pattern of resistance to other drugs.

In those cases in which no synergy was detected, there was no antagonism either. Even in these cases, addition of doxycycline might be helpful as an adjuvant in the prevention of emergence of resistance to other drugs, a principle that was already tested for this family of drugs [28].

The synergistic effect observed in some of the strains may be related to the fact that aminoglycosides and tetracyclines are

Table 4
Homoplasies detected by whole-genome sequencing.

Position in reference	Locus	Type	Substitution	Category	Reference base	SNP	H111740353	H111880072	H121860041	H111540004
7582	*gyrA*	Multi-SNP	D94A,G	Information pathway	A	C,G	.	C	.	.
50557	*ino1*	NS	R190G	Metabolism, respiration	T	C	C	C	C	C
75233	Intergenic	Intergenic	–	_	C	A	A	A	A	A
79558	Rv0071	NS	A25P	Information pathway	G	C	C	C	C	.
79560	Rv0071	S	A25A	Information pathway	C	G	G	G	G	.
761144	*rpoB*	NS	H445Y	Information pathway	C	T	.	.	.	.
761160	*rpoB*	NS	S450L	Information pathway	C	T	T	T	.	T
781692	*rpsL*	NS	K43R	Information pathway	A	G	G	G	.	.
1096573	Intergenic	Intergenic	–	_	A	G	G	G	G	G
1164577	Intergenic	Intergenic	–	_	A	G	.	.	.	.
1249270	Rv1125	S	A394A	Chp	G	C	.	.	.	.
1341303	Intergenic	Intergenic	–	_	A	G	.	.	.	.
1441584	Rv1288	S	L77L	Chp	T	C	.	.	.	.
1441587	Rv1288	NS	N78Y	Chp	A	T	.	.	.	.
1441590	Rv1288	NS	W79R	Chp	T	C	.	.	.	.
1481193	Rv1319c	NS	D439E	Metabolism, respiration	A	C	.	.	.	.
1673433	Intergenic	Intergenic	–	_	C	T	.	N	.	.
2122403	*lldD2*	NS	V253M	Metabolism, respiration	C	T	T	T	T	T
2123177	Intergenic	Intergenic	–	_	T	G	G	G	G	G
2155176	*katG*	NS	S315T	Virulence	C	G	G	G	G	G
2626608	Intergenic	Intergenic	–	_	G	A	.	.	.	.
2752706	Intergenic	Intergenic	–	_	C	A	A	A	A	A
3247324	*ppsA*	NS	D624E	Lipid metabolism	C	G	G	G	G	G
3593464	Intergenic	Intergenic	–	_	C	T	.	.	.	.
3626475	*mtrB*	S	L49L	Regulators	C	T	.	.	.	.
3743664	Intergenic	Intergenic	–	_	G	A	A	A	A	A
3906318	*lipF*	NS	R233C	Metabolism, respiration	G	A	A	A	A	A
4247436	*embB*	Multi-SNP	M306Q, V	Cell wall	A	C,G	G	G	.	.
4247438	*embB*	NS	M306I	Cell wall	G	A	.	.	A	.
4254353	Intergenic	Intergenic	–	_	T	A	.	.	.	.
4313163	Rv3839	NS	R131Q	Chp	G	A	.	.	.	.

Position in reference	H111620021	H112240073	H111040027	H121160050	H121320018	H111980010	H112160033	H111860011	H112040042	H111900039	H111620002
7582	.	.	.	.	N	.	G	.	.	.	.
50557	C	C	C	C	C	C	C	C	C	C	C
75233	A	A	N	A	A	A	A	N	A	A	A
79558	C	C	C	C	.	C	.	.	.	.	.
79560	G	G	G	G	.	G	.	.	.	.	.
761144	.	.	T	.	.	.	.	.	.	.	.
761160	T	T	.	T	T	.	T	T	.	.	.
781692	G	G	G	.	.	N	.	.	.	.	.
1096573	N	G	N	G	G	G	G	.	.	.	.
1164577	.	.	.	.	.	.	G	G	G	G	G
1249270	.	.	.	.	.	.	.	.	.	C	.
1341303	.	.	.	.	.	.	G	G	G	G	G
1441584	.	.	.	.	.	.	C	C	C	C	C
1441587	.	.	.	.	.	.	T	T	T	T	T
1441590	.	.	.	.	.	.	C	C	C	C	C
1481193	.	.	.	.	.	.	C	C	C	C	C
1673433	.	.	.	.	.	.	T	.	.	.	.
2122403	T	T	T	T	T	T	.	.	.	.	.
2123177	G	G	G	G	G	G	G	G	G	G	G
2155176	G	G	G	G	G	G	G	G	.	.	G
2626608	.	A	.	.	.	.	A	A	A	A	A
2752706	A	A	A	A	A	A	A	A	A	A	A
3247324	G	G	G	G	G	G	.	.	.	.	.
3593464	.	.	.	.	.	.	.	.	.	T	.
3626475	.	.	.	.	.	.	.	.	.	.	.
3743664	A	A	A	A	A	A	.	.	.	.	.
3906318	A	A	A	A	A	A	.	.	.	.	.
4247436	G	.	.	.	.	.	C	.	.	.	.
4247438	.	.	.	.	.	A	.	.	.	.	A
4254353	.	.	.	.	.	.	.	.	.	.	.
4313163	.	.	.	.	.	.	A	A	A	A	A

Position in reference	H112140033	H111840003	H111500010	H121920020	H112080012	H110860461	H111900041	H122020037	H120640724	H111760052	H112080018
7582	G	.	.	.	.	.	.	.	.	.	.
50557	C	C	.	.	.	.	.	C	.	C	C
75233	A	A	.	.	.	.	.	.	A	.	.
79558	.	.	.	.	.	.	.	.	.	.	.
79560	.	.	.	.	.	.	.	.	.	.	.
761144	.	T	.	.	.	.	.	.	T	.	.
761160	.	.	.	.	.	.	.	T	.	T	.
781692	.	.	.	.	.	.	.	.	.	.	.
1096573	.	.	.	.	.	.	.	.	.	.	.
1164577	G	G	.	.	.	.	.	G	.	.	.
1249270	.	.	C	C	C	.	.	.	.	.	.

Table 4 (*Continued*)

Position in reference	H112140033	H111840003	H111500010	H121920020	H112080012	H110860461	H111900041	H122020037	H120640724	H111760052	H112080018
1341303	G	G	G	G	G	.	.	.	.	.	.
1441584	C	C	.	.	.	.	.	.	.	C	.
1441587	T	T	.	.	.	.	.	.	.	T	.
1441590	C	C	.	.	.	.	.	.	.	C	.
1481193	C	C	.	.	.	.	C	.	C	C	C
1673433	.	.	T	T	T	.	.	.	T	.	.
2122403	.	.	T	T	T	.	.	.	.	.	.
2123177	G	G	G	G	G	G	G	G	.	G	G
2155176	G	.	.	.	.	.	.	G	.	G	.
2626608	A	A	A	A	A	.	.	.	.	.	.
2752706	A	A	A	A	A	A	A	.	A	A	A
3247324	.	.	G	G	G	G	G	G	G	G	G
3593464	.	.	.	.	.	.	.	.	.	.	T
3626475	.	.	.	.	.	.	.	.	.	T	T
3743664	.	.	N	N	A	.	.	.	.	.	.
3906318	.	.	N	N	A	.	.	.	.	.	.
4247436	G	.	.	.	.	.	.	.	.	G	.
4247438	.	.	.	.	.	.	.	.	.	.	.
4254353	.	.	.	.	.	.	.	.	A	A	.
4313163	A	.	.	.	.	.	.	.	.	A	.

both substrates for several efflux pumps present in *M. tuberculosis* [31–35]. Addition of doxycycline might compete for binding to the efflux pump, thus removing amikacin and preventing its extrusion, or vice versa. The different profiles observed here (synergy and indifference) could be related to different efflux pumps expressed in different strains, each with a preferred substrate amongst the two compounds.

In this study, no genotypic differences in expression of the genes coding these pumps and phenotype were detected. It was previously observed, however, that even though no difference could be detected between different phenotypes and efflux pump gene expression, clinical samples present a higher transcription level for these genes compared with non-clinical reference strains [36,37].

A mutation that confers streptomycin resistance in the gene for the ribosomal protein RpsL was associated with loss of the synergistic phenotype. Streptomycin and the tetracyclines all interfere with translation by inhibiting delivery of aminoacylated tRNA to the A-site of the ribosome [11]. The RpsL (Rv0682) mutation did not explain lack of synergy for all isolates, indicating that there is a multifactorial genetic basis for the phenotype.

Early reports for other members of the tetracycline family suggested that activity might be enhanced at a lower pH (pH 5.5) than the one used in this study (pH 6.8) [28]. If this is also true for doxycycline, it would make it a good candidate to be used alongside pyrazinamide, whose most potent action is also obtained at pH 5.5 [38], to act within the granuloma.

5. Conclusions

The combination of doxycycline and amikacin has either an indifferent or synergistic effect against *M. tuberculosis* strains.

Addition of doxycycline to an amikacin-containing regimen might be of benefit not only because of the intrinsic activity of the antibiotic but also by exerting an immunomodulatory effect, as previously reported [29], provided that it is tolerated by the patient.

Exploration of strategies such as synergy and pharmacokinetics is of paramount importance in trying to prevent the emergence of resistance.

As current 6-month rifampicin-based regimens are extremely effective and we did not examine the rate of activity specifically, it is unlikely that an amikacin (aminoglycoside)–doxycycline combination would be used outside the treatment of highly drug-resistant cases. As we saw no antagonism, the routine addition of doxycycline to MDR-TB regimens containing amikacin is likely to be helpful.

All patients with evidence of significant lung damage (e.g. late diagnosis) might benefit from doxycycline immunomodulatory activity alongside standard TB therapy, as well as MDR/XDR-TB patients.

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