ORIGINAL ARTICLES

Study of Promoter and Structural Gene Sequence of *whiB7* in MDR and XDR forms of *Mycobacterium tuberculosis*

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ABSTRACT

Resistance phenomenon in M tuberculosis *is mainly based on decreased permeability of the bacterial envelope and function of effluent pumps. The regulatory gene of the whiB7 transcription determines drug resistance in these bacteria. Increases in* WhiB7 *protein activity induce transcription of resistance genes leading to intrinsic multidrug resistance. The aim of this work was to evaluate the whiB7 gene sequence in susceptible, MDR and XDR clinical isolates of* M tuberculosis *in order to further design an inhibitor. Thirty-three clinical isolates of MTB identified as susceptible, MDR and XDR-TB were investigated by PCR for sequencing of the entire promoter (429 bp), structural gene (279 bp) and the end of the upstream gene uvrD (265 bp). No differences were detected in the sequences of the structural gene in susceptible and MDR with XDR isolates and all of them terminated at TGA as stop codon. Examination of sequence profiles of the promoter part of whiB7 by several sets of primers proved that there were no differences between sequence of susceptible, MDR and XDR isolates by type strain (H37Rr). Furthermore, the structure of* WhiB7 *protein was studied in achieved sequences from clinical isolates. We found that the promoter and structural gene of whiB7 are highly conservative in clinical susceptible and resistant isolates. It is a key finding that would assist in the design of an inhibitor for the* WhiB7 *protein in all clinical forms in further studies.*

Keywords: *whiB7*, *M tuberculosis*, sequence, MDR, XDR

Estudio del Promotor del ADN y la Secuencia de Genes Estructurales de *whiB7* en las Formas MDR y XDR de *Mycobacterium tuberculosis*

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RESUMEN

El fenómeno de resistencia en M tuberculosis *se basa principalmente en la disminución de la permeabilidad de la envoltura bacterial y la función de las bombas efluentes. El gene regulador de la trascripción de whiB7 determina la resistencia al medicamento en estas bacterias. Los aumentos en la actividad de proteína de* WhiB7 *inducen la trascripción de genes de resistencia que llevan a la resistencia intrínseca de multimedicamentos. El objetivo de este trabajo fue evaluar la secuencia de genes de whiB7 en aislados clínicos susceptibles MDR y XDR de* M tuberculosis *para mejorar el diseño de un inhibidor. Treinta y tres aislados clínicos de MTB identificados como MDR y XDR-TB susceptibles, fueron investigados por PCR para la secuenciación del promotor entero (429 bp), el gene estructural (279 bp) y el extremo del uvrD gen arriba (265 bp). No se detectó diferencia alguna en las*

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secuencias del gene estructural en aislados susceptibles, MDR y XDR, terminando todos ellos en TGA como codón de terminación.
El examen de perfiles de la secuencia de la parte de promotor de whiB7 por varios conjuntos de iniciadores (primers), demostró que no había ninguna diferencia entre la secuencia de aislados susceptibles MDR y XDR por tipo de cepa (H37Rv). Además, la estructura de la proteína de WhiB7 *se estudió en secuencias logradas de aislados clínicos. Se encontró que el promotor y el gene estructural whiB7 son muy conservadores en aislados clínicos susceptibles y resistentes. Se trata de un hallazgo clave que ayudaría a designar un inhibidor para la proteína* WhiB7 *en todas las formas de este patógeno en estudios ulteriores.*

Palabras claves: *whiB7*, *M tuberculosis*, secuencia, MDR, XDR

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INTRODUCTION

Drug resistance in tuberculosis (TB) is a matter of great concern for TB control. MDR-TB is defined as resistance to at least rifampicin (RIF) and isoniazid (INH). XDR-TB is TB showing resistance to at least rifampicin and isoniazid in addition to any fluoroquinolone, and to at least 1 of the 3 following injectable drugs used in anti-TB treatment: capreomycin, kanamycin and amikacin (1, 2, 7). This resistance is partially provided by the low permeability of their unique cell envelope (3).

Ligation of mycolic acids to structural components of the mycobacterial cell wall generates a hydrophobic, impermeable barrier that provides resistance to toxic compounds such as antibiotics (4, 5). There is a complementary system in *Mycobacterium tuberculosis* (*M tuberculosis)* that coordinates resistance to drugs that have penetrated the envelope, allowing mycobacteria to tolerate diverse classes of antibiotics that inhibit cytoplasmic targets. This system depends on *whiB7*, a gene in pathogenic *Mycobacterium* that was found to be a central regulator that coordinates the expression of a family of resistance genes able to inactivate antibiotics that have penetrated into the cytoplasm. In *M tuberculosis*, *whiB7* is induced by sub-inhibitory concentrations of antibiotics (erythromycin, tetracycline and streptomycin) and *whiB7* null mutants are hypersusceptible to antibiotics *in vitro*. In addition to antibiotics, *whiB7* is induced by fatty acids that pathogenic *Mycobacterium* species may accumulate internally within eukaryotic hosts during infection (3−6).

There are 14 genes belonging to the *whiB* family. Site-directed mutagenesis of this set of genes has shown that the multidrug susceptible phenotype is a unique characteristic of *whiB7* mutants of *Streptomyces coelicolor* [*S coelicolor*] (3). BLAST searches did not identify orthologs in any other published bacterial genome sequences beside actinomycetes.

Gene expression profiling analyses demonstrate that *whiB7* transcription determines drug resistance by activating expression of a regulon including genes involved in ribosomal protection and antibiotic efflux (3, 5). Increases in *whiB7* activity induce transcription of resistance genes leading to intrinsic multidrug resistance to macrolides (MCs), lincosamides (LSs), TE and aminoglycosides (AGs), and other unknown responses that are coordinated with metabolic adaptations (5).

Therefore, since a single regulatory protein (WhiB7) activates resistance to multiple drugs in mycobacterial pathogens, components of the whiB7 system could serve as novel drug targets (*ie* inhibitors), rendering *M tuberculosis* or multidrug-resistant derivatives more antibiotic-susceptible (3−6).

Thus, it is necessary to determine conservativity of *whiB7* gene in clinical isolates that may render inhibitor unsuccessful or interfere with designing of an inhibitor for all clinical forms of MTB.

The aim of this study was to evaluate any probable occurrence of mutations in the promoter and structural gene *whiB7* in susceptible, MDR and XDR clinical isolates of *M tuberculosis*.

MATERIALS AND METHODS

In this study, 33 clinical strains of *M tuberculosis* were isolated from sputum of patients suffering from tuberculosis (10). These isolates were obtained from different patients with no familial ties. All patients were reviewed for clinical diagnostic criteria for tuberculosis: weight loss, cough, PPD, chest X-ray *etc*. Patient sputum samples were cultured on Löwenstein-Jensen medium and grown colonies were identified to the species level using TCH (2-thiophene carboxylic acid) and PN99B (paranitrobenzoic acid) selective media (7−9) or by standard biochemical procedures. Catalase activity was assayed using a mixture of hydrogen peroxide (15%) and Tween 80 (10%). *M tuberculosis* H37Rv was included as a susceptible control strain in the drug-susceptibility testing and also as a positive control for the catalase assay.

Susceptibility testing was performed by the absolute concentration method on Löwenstein-Jensen medium using the critical drug concentrations. A microbial suspension containing 5×10^8 organisms/ml was prepared according to McFarland turbidity standards and was diluted 1:10; then, 0.2 ml of the dilution was added to Löwenstein-Jensen medium with or without a drug. The culture tubes were incubated at 37°C, and growth was monitored after three weeks of incubation and assessed as described by WHO (World Health Organization, 1998). An isolate was considered resistant

when bacterial growth occurred in the presence of a concentration of Rifampicin (RIF) 40 mcg/ml, Isoniazid (INH) 0.2 mcg/ml, Ethambutol (EMB) 2 mcg/ml, Streptomycin (SM) 4 mcg/ml (as First Line Drugs), and Kanamycin (K) 30 mcg/ml on slants with H37Rv strain of *M tuberculosis* as the positive control, using the BACTEC system in level III laboratory. According to the recommendations of WHO, those strains which were resistant to at least INH and RIF were tested for their susceptibility to any fluoroquinolone and at least one of three injectable second-line drugs (capreomycin, kanamycin and amikacin) in the BACTEC system for detection of XDR isolates. This definition of XDR-TB was agreed to by the WHO Global Task Force on XDR-TB [WHO Stop TB Department, 2006] (1).

Purification of *M tuberculosis* genomic DNA was performed using Genomic DNA Purification Kit (Fermentas KO512). On the other hand, Chelex 100 method [Walsh, 1991] (14) was accomplished by heating of two colonies of isolate dissolved in TAE buffer [Distilled Water (DW) with 18.2 MΩ cm] in 5% Chelex 100 for 45 minutes at 95°C followed by centrifugation for 15 minutes at 14 000 rpm (3 times) for total removal of Chelex 100 that would interfere with the polymerase chain reaction [PCR] (8, 9, 16).

Polymerase chain reaction analysis was accomplished using oligonucleotide primers that were designed from the *M tuberculosis* H37Rv genome sequence, by help of several server programmes (12) [Table 1].

Polymerase chain reaction with 6 sets of primers was carried out in 50 µl containing 1 µl dNTP, 5 µl Buffer (with MgCl2), 1U*Taq* polymerase, 25 pmol of each primer and 1−5 µl of DNA template. Annealing temperature for each pair of primers was calculated by means of online programmes. Conditions for PCR are revealed in Table 1. Polymerase chain reaction products containing these genes were detected by 1.5% agarose-ethidium bromide gel electrophoresis, followed by UV detection then they were purified from agarose gel after electrophoresis using a DNA extraction kit (Fermentas KO513) according to the manufacturer's instructions. Extracted DNA concentrations were measured by Nucleic Acid analyzer (DU 730, Life Science UV/V spectrophotometer).

Extracted and measured DNA samples were amplified in a Rotor-Gene (RG-3000, Corbett Research Inc.) by Thermo-Sequenase Cy5 Dye Terminator Sequencing Kit (GE Healthcare 27-2682-01) at conditions specified for each primer as shown in Table 1 and were sequenced directly using an automatic DNA sequencer (Amersham auto sequencer). For avoidance of any mistake, some of the sequences experiments were repeated 2−3 times. On the other hand, forward and reverse primers were used for sequencing.

Table 1: Primers used for PCR amplification of *whiB7* gene (the first cycle was at 95°C for 3 min and the last cycle at 72°C for 10 min)

Sets	DNA targets	Direction	Primers (5'-3')	Product size (bp)	Parameters for PCR	Parameters for sequence
1	*whiB7*	F	CAGACAAAGATTGCCGGTTT	279	95°C,15s	95°C,15s
		R	TCGAGCCTTGGTCGAATATC		55°C, 15s	55°C, 15s
					72°C, 40s,	72°C, 30s,
					40 cycles	35 cycles
2	w686	F	AAGCTTATCGATGGTGTGAGACGTGTGCAGC	686	94°C, 30s	95°C, 30s
		R	TCCGCGCAAGGATGCTGTTGCATAGTCTAGATC		57°C, 30s	57°C, 30s
					72°C, 30s	72°C, 80s
					40 cycles	30 cycles
3	w973	F	AAGCTTATCGATGGTGTGAGACGTGTGCAGC	973	94°C, 30s	95°C, 30s
		R	CCGCGCAAGGATGCTGTTGCATAGTCTAGATC		57°C, 30s	57°C, 30s
					72°C, 30s	72°C, 80s
					35 cycles	30 cycles
4	w667	F	AGCTGCTGCCACCGGTTAAC	667	94°C, 30s	95°C, 30s
		R	CCGCGCAAGGATGCTGTTGCATAGTCTAGATC		57°C, 30s	57°C, 30s
					72°C, 30s	72°C, 80s
					35 cycles	30 cycles
5	wt1	F	TTAACCTCCAGGTCGCATTCTGCT	236	94°C, 30s	95°C, 30s
		R	GAAAGTTTGGCCACGGATCCTGT		56°C, 30s	56°C, 30s
					72°C, 30s	72°C, 80s
					35 cycles	30 cycles
6	wt2	F	AGGTCAGAAAATCGGTTGTGGTCAGC	329	94°C, 30s	95°C, 30s
		R	TGGTGGCGGTTCTTCGAAAGTGAT		56°C, 30s	56°C, 30s
					72°C, 30s	72°C, 80s
					35 cycles	30 cycles

Fragments that were used for whole screening of *whiB7* in clinical isolates were: 1) *whiB7* structural gene (called as *whiB7*); 2) last part of uvrd2 including promoter of *whiB7* (w686); 3) w686 plus structural gene (w973); 4) a part of the promoter plus structural gene (w667); 5) wt_1, a part of the promoter; 6) wt_2, a part of the promoter (Table 2 and Fig 1).

Table 2: Fragments were used for whole screening of Open Reading Frame (ORF) of *whiB7* gene

Sets	DNA targets	Location in ORF of *whiB7* (708 bp) and uvrD2	Types of fragments (bp)	Length
1	*whiB7*	429 – 708	structural gene	279
2	w686	-257 to 429	uvrd2 & promoter	686
3	w973	-257 to 708 (+8)	w686 plus structural gene	973
4	w667	49 to 708 (+8)	A part of promoter plus structural gene	667
5	wt1	344 – 580	A part of promoter	236
6	wt2	309 – 638	A part of promoter	329

Analysing the results of sequencing the *regulatory* region consisting of promoter and gene *whiB7* (Fig. 2) was accomplished using the ALFwin Sequence Analyser module V2, Mega4, NCBI-BLAST and BLASTP, DNAMAN (Quebec, Canada) and BioEdit programmes. Protein sequence of isolates was got from translation of nucleotide sequences in a Mega programme (11).

RESULTS

Thirty-three clinical isolates were determined as *M tuberculosis*. Drug susceptibility testing revealed that 12 isolates were MDR, 15 XDR and six susceptible *M tuberculosis* (as controls). DNA extraction by Chelex 100 was the best material for purification and maintenance of DNA over a long time.

The results showed that the probes designed in this study are specific and have the best quality. Using several primers for different DNA targets allowed a good opportunity for carefully screening all parts of ORF *whiB7* (Fig. 1).

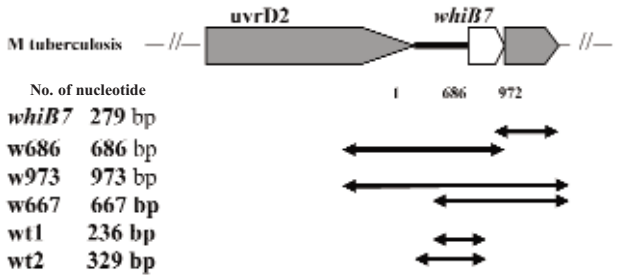

Fig. 1: Positions of studied fragments in ORF of *whiB7*.

All isolates were screened for mutations at the regulatory region including the promoter and structural gene regions.

Sequencing results of four susceptible, six MDR and 15 XDR clinical isolates of *M tuberculosis* were accomplished and were repeated by another or the same primer to avoid bias. All of them terminated by stop codon TGA and we did not find any mutation in the structural gene of *whiB7* in the clinical isolates. These are more obvious when translation of nucleotides to amino acids by proper programme was done.

Results of sequencing of *whiB7* in all studied isolates revealed that there was not any mutation in the promoter region of *whiB7*. We used several different sets of primers to avoid any obvious errors and found that the structural gene *whiB7* is highly conservative in clinical susceptible and resistant isolates. It confirmed the fact that *whiB7* activity causes multidrug resistance in resistant XDR and MDR isolates.

A comparison study of protein WhiB7 in all cases performed by translation of the sequences in a Mega programme, revealed the typical structure of this gene in clinical isolates. This protein consists of 93 amino acids (279 nucleotides). As was aligned in Fig. 2, four conserved cysteines (Cys-X22-Cys-X2-Cys-X5-Cys) were clearly visible. There were no mutations especially in these four cysteines.

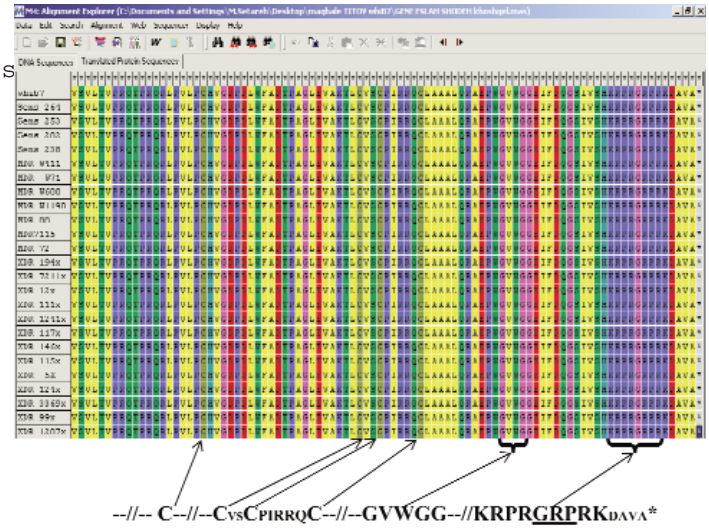

Fig. 2: Structure of protein WhiB7 in clinical isolates: four cysteines as Cys-X22-Cys-X2-Cys-X5-Cys, Conserved HTH-like motif characterized by the residues signature G-(V)-W-G-G and C-terminal "AT- Hook", clearly visible.

Glycine-rich sequence, as in all members of the *whiB* family in actinomycets, consists of a HTH-like motif characterized by the 7-residue signature G-(VI)-W-G-G. In our clinical isolates and strain type of *M tuberculosis* H37RV,

a HTH-like motif **(GVWGG)** was shown.

A typical AT-Hook DNA binding consensus sequence that was found only in WhiB7 paralogs as a core, consists of

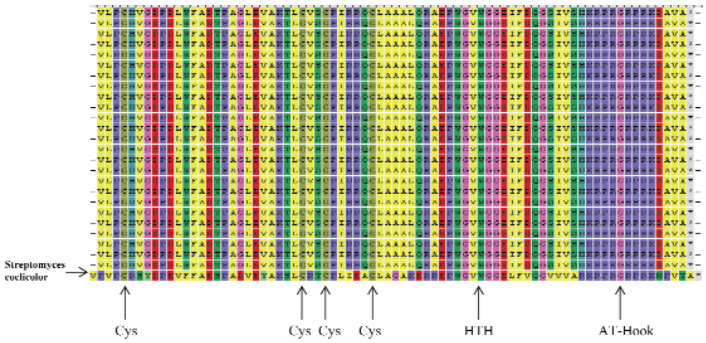

Fig. 3: Comparison of structure protein WhiB7 in Streptomyces (123 amino acids) with resistant clinical isolates of *Mycobacterium tuberculosis* (93 amino acids). Four cyctein, HTH-like motif and AT-Hook obviously identical (in the same place).

GRP surrounded by an arginine-rich domain (Figs. 2, 3).

It is of note that only these three characters without any difference, common among our susceptible, MDR and XDR clinical isolates, standard strain of *M tuberculosis* H37RV and *Streptomyces coelicolor* (4, 13, 14) could be detected.

DISCUSSION

The intrinsic resistance of *Mycobacterium tuberculosis* has traditionally been attributed to the unusual multi-layer cell envelope that functions as an effective barrier to the penetration of antibiotics (5).

whiB7 is different from systems reported in other bacteria that allow adaptation to a variety of different nonspecific stress conditions and may provide multi-resistance. Function of this gene is also unique in that it confers relatively high levels of resistance. The presence of structural genes and corresponding regulatory systems in *Mycobacterium* suggests that this system provides selective advantage (3). Previous research revealed no *whiB* family-like genes in organisms other than actinomycetes (14).

The WhiB7 protein signature is defined by:

* Four absolutely conserved cysteines that form an oxygen susceptible iron sulfur cluster arranged as Cys-X14–22-Cys-X2-Cys-X5-Cys.
* A tryptophan (abbreviated as Trp or W) within a glycine-rich sequence (GVWGG), as a conserved HTH-like motif.
* In addition, *whiB7* paralogs also encode a C-terminal ‘‘AT- Hook’’ domain (3, 15).

In proteins, the helix-turn-helix (HTH) is a major structural motif capable of binding DNA. It is composed of two α helices joined by a short strand of amino acids and is found in many proteins that regulate gene expression. In WhiB7, although the HTH motif does not comprise a typical HTH motif (15), it is believed to be involved in DNA binding (14). The present study showed an HTH-like motif as a GVWGG protein sequence of WhiB7 in resistant clinical isolates (Figs. 2, 3). This motif is in some members of the *whiB* family as GIWGG (14).

Secondary structure analysis of *whiB* indicated a potential helix±loop±helix structure from residues 64 to 84. The importance of this region was underscored by the fact that the *whiB70* mutant, which contains a missense mutation leading to substitution of Leu (by Pro) had the same phenotype as a *whiB* deletion mutant (14).

whiB7 paralogs also encode a C-terminal ‘‘AT- Hook’’ domain that is known to bind AT-rich DNA sequences (3). As shown in Figs. 2 and 3, this domain shows no differences between the clinical isolates and sequence of *Streptomyces coelicolor*.

CONCLUSION

This work is the first study on the sequence of *whiB7* gene in susceptible, MDR and XDR clinical isolates. We did not find any mutation in this gene in any clinical isolate and it was revealed that the promoter and structural gene of *whiB7* are highly conservative in XDR and MDR isolates.

These findings have confirmed the need to design a proper inhibitor for the WhiB7 protein in all clinical forms (susceptible, MDR, XDR).

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REFERENCES

1. Palomino JC, Cardoso Leão S, Ritacco V (Eds). Tuberculosis 2007: from basic science to patient care: 2007. Available from: www.TuberculosisTextbook.com
2. CDC, Centers for Disease Control and Prevention. Extensively Drug-Resistant Tuberculosis (XDR TB),www.cdc.gov/tb, July 2007, 1−3.
3. Morris RP, Nguyen L. Ancestral antibiotic resistance in Mycobacterium tuberculosis, PNAS. 2005; **102:** 12200–05.
4. Thopmson CJ, Grzesiek S. Overcoming intrinsic multidrug resistance to discover and expand the repertoire of antibiotics active against mycobacterial pathogens. 4049-069384, 01.10.2002-31.03.2006. NRP 49 final report, 1−2.
5. Nguyen L, Thompson CJ. Foundations of antibiotic resistance in bacterial physiology: the mycobacterial paradigm, TRENDS in Microbiology, 2006; **14:** 304−12.
6. Soni V, Agrawal P, Khandrika LP. Reporter gene based method for the screening of anti-tuberculosis drugs by using essential and regulatory genes of mycobacteria as drug target, United States Patent: 6, 645. November 11, 2003.
7. Setareh M, Titov LP, Surkova LK. High level association of mutation in KatG315 with MDR and XDR clinical isolates of *Mycobacterium tuberculosis* in Belarus. Acta Microbiologica et Immunologica Hungarica 2009; **56:** 313–25.
8. Arjomandzadegan M, Owlia P, Ranjbar R, Farazi AA, Sofian M, Sadrnia M et al. Prevalence of mutations at codon 463 of *katg* gene in mdr and xdr clinical isolates of *mycobacterium tuberculosis* in Belarus and application of the method in rapid diagnosis. Acta Microbiologica

Et Immunologica Hungarica 2011; **58:** 51–63.

9. Arjomandzadegan M, Owlia P, Ranjbar R, Farazi AA, Sofian M, Sadrnia M et al. Rapid and simple approach for identification of *Mycobacterium tuberculosis* and *M. bovis* by detection of regulatory gene *whiB7.* Acta Microbiologica et Immunologica Hungarica 2011; **58:** 65–73.
10. Kent PT, Kopica GP. Public health mycobacteriology: A guide for the level III laboratory. CDC, U.S. Dept. of Health and Human Services, Publication number (CDC) 86-216546, Atlanta 1985, 21–30.
11. Nollau P, Wagener C. Methods for detection of point mutations: performance and quality assessment**,** Clinical Chemistry 1997; **43:** 1114–28.
12. Yuryev A. PCR Primer Design Part I: Basic principles and software. In: Methods in molecular biology. Humana Press, Springer; 2006.
13. Fleischmann RD, Alland D, Eisen JA, Carpenter L, White O, Peterson J et al. Whole-Genome Comparison of *Mycobacterium tuberculosis* Clinical and Laboratory Strains. J Bacteriology 2002; **184:** 5479–90.
14. Soliveri JA. Multiple paralogous genes related to the *Streptomyces coelicolor* developmental regulatory gene *whiB* are present in *Streptomyces* and other actinomycetes. Microbiology 2000; **146:** 333–43.
15. Raghunand TR, Bishai WR. Mapping essential domains of Mycobacterium smegmatis WhmD: Insights into WhiB Structure and Function. J Bacteriology 2006; **188:** 6966−76.
16. Walsh PS, Metzger DA, Higuchi R. Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. Biotechniques 1991; **10:** 506−13.