Detection of *Mycobacterium tuberculosis* complex and non-tuberculous mycobacteria by multiplex polymerase chain reactions

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اكتشاف مركب متفطرات السلّ والمتفطرات غير السلية بتفاعلات سلسلة البوليمراز المتعددة

أبو سالم مصطفى وطاهر عدنان أبل وتلسي داس شوج

خلاصة: تم تقييم مدى قدرة تفاعلات سلسلة البوليمراز المتعددة ذات الشريطين وذات الشرائط الثلاثة، على اكتشاف مركب متفطرات السل والمتفطرات غير السلية والتفريق فيما بينهما. ولقد استطاعت تفاعلات سلسلة البوليمراز أن تفرق بين المتفطرات السلية وغير السلية عند اختبار الذراري المعيارية للمتفطرات السلية ومستفرداتها المعزولة سريرياً. وتبيَّن أن نسبة حساسية الطرائق ذات الشريطين وذات الشرائط الثلاثة في اكتشاف المتفطرات السلية في العينات السريرية مقارنة بفحوص اللطاخات أو المزارع أو كليهما كانت تبلغ 88% و75% على التوالي. ومع أن نسبة نوعية هذه الطرائق جميعها بلغت 100% إلا أن تميُّز حساسية الطريقة ذات الشريطين يوحي بأنها يمكن أن تكون أكثر نفعاً في تشخيص السل وفي التفريق بين مركب متفطرات السل وبين المتفطرات غير السلية.

ABSTRACT The ability of two-band and three-band multiplex polymerase chain reactions to detect and differentiate *Mycobacterium tuberculosis* complex from non-tuberculous mycobacteria was evaluated. The polymerase chain reactions differentiated between *M. tuberculosis* and non-tuberculous mycobacteria when standard strains and clinical isolates of mycobacteria were tested. The sensitivity of the two-band and three-band techniques to detect *M. tuberculosis* in clinical specimens, compared with smear and/or culture, was 88% and 75% respectively. Although both techniques showed 100% specificity, the superior sensitivity of the two-band technique suggests that it could be more useful in the diagnosis of tuberculosis and in differentiating *M. tuberculosis* complex from non-tuberculous mycobacteria.

Identification de Mycobacterium tuberculosis *du complexe* tuberculosis *et des mycobactéries non tuberculeuses par PCR multiplex*

RESUME On a évalué la capacité des techniques PCR de co-amplification à identifier et différencier Mycobacterium tuberculosis des mycobactéries non tuberculeuses. Les fragments d'ADN provenant de gènes à copies multiples de M. tuberculosis ont été amplifiés. L'amplification génique a permis de différencier entre M. tuberculosis et des mycobactéries non tuberculeuses lorsque des souches standard et des isolements cliniques de mycobactéries ont été analysés. La sensibilité des deux techniques de co-amplification (deux bandes et trois bandes) pour la détection de M. tuberculosis dans les échantillons cliniques, comparée à la culture et/ou l'examen microscopique était de 88% et 75% respectivement. Bien que ces deux techniques aient montré une spécificité de 100%, la sensibilité supérieure de la première (deux bandes) laisse penser qu'elle pourrait être plus utile dans le diagnostic de la tuberculose et dans la différenciation de M. tuberculosis du complexe tuberculosis des mycobactéries non tuberculeuses.

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Introduction

Tuberculosis is a communicable disease with significant morbidity and mortality. The diagnosis of tuberculosis is largely based on conventional approaches, which rely on clinical features and the results of microscopy and culture. Clinical features are not generally diagnostic and microscopy, while rapid, lacks sensitivity (detection limit = 7.5×10^3 organisms/ml) and specificity. Culture methods are sensitive (detection limit = 10 organisms/ml) and specific but they are slow. Several alternative approaches have been attempted for the rapid and specific diagnosis of tuberculosis [*1*], but the molecular methods, especially polymerase chain reaction (PCR) assays, are the most promising.

PCR for the detection of *Mycobacterium tuberculosis* in clinical specimens requires 1–2 days and has a specificity and sensitivity comparable to culture methods [*2–6*]. Infections caused by non-tuberculous mycobacteria (NTM) are increasing in immunocompromised individuals, e.g. AIDS patients [*7,8*], and effective therapeutic regimens are different for patients infected with *M. tuberculosis* or NTM [*9*]. It is necessary therefore to establish and evaluate PCR assays which can differentiate between these two groups of mycobacteria. This can be achieved by simultaneous amplification of two or more DNA targets, some specific to *M. tuberculosis* and others common to all mycobacteria in multiplex PCRs (MPCRs). In order to establish sensitive MPCRs, we selected DNA targets in genes present in multiple copies in the *M. tuberculosis* genome, i.e. insertion sequences IS6110 (6 to 17 copies in most isolates) [*10*], IS1081 (5 to 6 copies) [*11*] and the gene encoding antigen (Ag) 85 complex (3 copies) [*12*]. MPCRs were first established with culture-grown mycobacteria and then applied in the detection of *M. tuberculosis* complex and NTM in culture isolates and directly in clinical specimens.

Materials and methods

Bacterial strains

The standard mycobacterial species and strains used in this study were obtained from American Type Culture Collection (ATCC), Rockville, Maryland, USA. These were: *M. tuberculosis* H37Ra ATCC 25177, H37Rv ATCC 25618, *M. bovis* BCG ATCC 19015, *M. africanum* ATCC 25420, *M. microti* ATCC 11152, *M. ulcerans* ATCC 19423, *M. kansasii* ATCC 12478, *M. marinum* ATCC 927, *M. simiae* ATCC 25275, *M. asiaticum* ATCC 25274, *M. scrofulaceum* ATCC 19981, *M. szulgai* ATCC 23069, *M. gordonae* ATCC 14470, *M. flavescens* ATCC 14474, *M. xenopi* ATCC 19970, *M. avium* ATCC 23070, *M. intracellulare* ATCC 13209, *M. gastri* ATCC 15754, *M. nonchromogenicum* ATCC 19530, *M. terrae* ATCC 15755, *M. triviale* ATCC 23292, *M. fortuitum* ATCC 6841, *M. chelonei* ATCC 14472, *M. phlei* ATCC 11758, *M. smegmatis* ATCC 19420, *M. vaccae* ATCC 15483, *M. chitae* ATCC 19627 and *M. diernhoferi* ATCC 19340. The non-mycobacterial species used were: *Escherichia coli, Clostridium diphtheria, Haemophilus influenzae, Nocardia astroides and Streptococcus* Group A. They were from the bacterial stock of the Department of Microbiology, Faculty of Medicine, Kuwait University, Kuwait.

Clinical specimens

A total of 151 clinical specimens sent for routine mycobacteriology to the Chest Diseases Hospital Laboratory, Kuwait were tested in this study. The specimens included 64 sputa, 31 cerebrospinal fluid, 10 lymph

node aspirates, 26 pleural, ascitic and pericardial fluids, 4 bronchial lavage, 6 urine, 5 pus and 5 tissue biopsies. After processing for routine mycobacteriology, each specimen was divided into two parts: one part was used for smear and culture and the other was processed for PCR. The smears were examined for acid-fast bacilli after staining with Ziehl–Neelsen stain and graded according to standard procedures [*13*]. The cultures were performed in the BACTEC radiometric culture system (Becton Dickinson and Company, New Jersey, USA). To differentiate between *M. tuberculosis* and NTM, inoculations were made in media bottles with or without P-nitro-α-acetylamine-β-hydroxypropiophenone (NAP) according to the manufacturer's instructions.

Oligonucleotide primers for amplification of target DNA

Six sets of oligonucleotide primers were used to amplify target DNA from the multiple copy genes encoding the antigen 85 complex [*14*], IS6110 [*15–18*] and IS1081 [*19*]. The designation and sequence of these primers are given in Table 1. The primers were synthesized and cartridge purified by Genosys Biotechnologies Incorporated, Cambridge, England. Using *M. tuberculosis* DNA as a template, the primer pairs MD1-MD2, KD1-KD2, HM1-HM2, SA1-SA2, SM1-SM2 and SK1-SK2 were expected to amplify 162 bp, 123 bp, 245 bp, 375 bp, 580 bp and 248 bp DNA, respectively [*14–19*].

DNA extraction procedures from cultures and clinical specimens

Single colonies of bacteria from cultures on solid media were suspended in 200 µl TE (10 mM Tris, pH 8.0 and 1 mM EDTA). The mycobacterial species and strains supplied by ATCC in lyophilized form were rehydrated in 1 ml TE. To release bacterial DNA into solution, the bacterial suspen-

Table 1 **Designation and sequence of the primers and size of the DNA amplified from multiple copy genes of *M. tuberculosis***

Primer designation	Nucleotide sequence	Size of amplified DNA	Target gene	Reference
MD1	5' ATCAACACCCCGGCGTTCGAG 3'	162 bp	Antigen 85 complex	*14*
MD2	5' CGGCAGCTCGCTGGTCAGGA 3'			
KD1	5' CCTGCGAGCGTAGGCGTCGG 3'	123 bp	IS6110	*18*
KD2	5' CTCGTCCAGCGCCGCTTCGG 3'			
HM1	5' CGTGAGGGCATCGAGGTGGC 3'	245 bp	IS6110	*15*
HM2	5' GCGTAGGCGTCGGTGACAAA 3'			
SA1	5' CTGGCGGGTCGCTTCCACGA 3'	375 bp	IS6110	*17*
SA2	5' TTCGACCGGCGGGACGTCGC 3'			
SM1	5' GGACAACGCCGAATTGCGAAGGGC 3'	580 bp	IS6110	*16*
SM2	5' TAGGCGTCGGTGACAAAGGCCACG 3'			
SK1	5' ACAGGCGAGCCCGGATCTGCTG 3'	248 bp	IS1081	*19*
SK2	5' GTTCAGCTCGCTTGCGGCGCTG 3'			

sions were heated at 95 °C for 15 minutes; 5 µl of the supernatants were used for PCR.

After processing for routine mycobacteriology, the clinical specimens were centrifuged at 10 000 RPM for 10 minutes. The pellets were washed twice and DNA was isolated using standard procedures of phenolchloroform extraction and ethanol precipitation [*10*]. DNA pellets were dried, suspended in 50 µl TE and 5 µl were used for PCR. To check for contamination during sample processing, control tubes without clinical specimens were processed in the same way as the tubes with clinical samples and served as extraction controls.

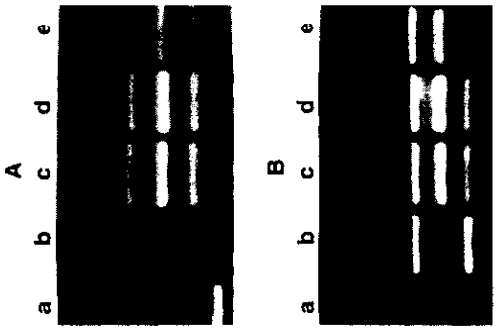
Amplification and analysis of target DNA

Target DNA was amplified using the oligonucleotide primers described in Table 1 and GeneAmp PCR reagent kits (Perkin-Elmer, Connecticut, USA) according to the manufacturer's instructions. Unless otherwise stated, each reaction mixture contained 4 mM $MgCl_2$, 2.5% dimethyl sulfoxide (DMSO), 100 ng of each relevant primer and 2.5 U of AmpliTaq DNA Polymerase in a total volume of 100 µl. Amplifications were carried out in thin-wall reaction tubes (Perkin-Elmer) using an automated thermal cycler (Perkin-Elmer, GeneAmp PCR System 9600). The samples were run for a total of 35 cycles. Each cycle consisted of two steps — step 1: 95 °C for 45 seconds, step 2: 70 °C for 120 seconds. To avoid false positives, separate physical facilities were used for sample preparation, amplification and analysis of the amplified products. In addition, positive and negative controls were included in all the experiments with clinical specimens. Positive controls contained 1 pg of *M. tuberculosis* DNA isolated by a standard phenol-chloroform extraction procedure [*10*]. For each batch of amplification with clinical isolates and specimens, DNA extraction controls and controls free of DNA were included.

Aliquots of amplified samples (15 µl) were loaded on 2% agarose gels in Tris-acetate-EDTA (TAE) buffer and subjected to electrophoresis in mini gel boxes for 30 minutes at 80 volts. The gels were stained with ethidium bromide at 0.5 µg/ml, observed under ultraviolet light for specific DNA bands and photographed. The DNA bands were identified according to size by comparing with the molecular weight marker (123 bp DNA ladder) loaded in a separate lane.

Results

In order to establish the most sensitive MPCR, we amplified target DNA from three genes present in multiple copies on the *M. tuberculosis* genome — IS6110, IS1081 and Ag85 complex. Four different DNA fragments from IS6110 (123 bp, 245 bp, 375 bp and 580 bp DNA) were amplified using appropriate primers (Table 1) and genomic DNA from *M. tuberculosis*. The results showed that the detection of 123 bp and 245 bp DNA fragments required genomic DNA from a minimum of 0.2 organisms of *M. tuberculosis*, whereas the detection limit for 375 bp and 580 bp DNA was 2 and 20 organisms, respectively (data not shown). Although amplifications of 123 bp and 245 bp DNA from IS6110 were the most sensitive, 123 bp DNA was not amplified in MPCR. Similarly, amplification of 580 bp DNA was not attempted. Attempts were made to establish three-band MPCR by targeting 375 bp, 248 bp and 162 bp DNA from IS6110, IS1081 and the gene encoding antigen 85 complex, respectively, and two-band MPCR by amplifying 245 bp and 162 bp DNA from IS6110 and the gene encoding antigen 85 complex,



1A Lanes:
a = molecular weight marker (123 bp DNA ladder)
b = 1.5 mM $MgCl_2$
c = 4.0 mM $MgCl_2$
d = 6.5 mM $MgCl_2$
e = 11.5 mM $MgCl_2$

1B Lanes:
a = molecular weight marker (123 bp DNA ladder)
b = 0% DMSO
c = 2.5% DMSO
d = 5% DMSO
e = 10% DMSO

Figure 1 **Effect of $MgCl_2$ (A) and DMSO (B) concentrations on the amplification of the target DNA amplified by the primer pairs SA1-SA2, SK1-SK2 and MD1-MD2 in three-band MPCR.**

respectively. Figure 1 shows the results for three-band MPCR at different concentrations of $MgCl_2$ and DMSO. The detection limit for *M. tuberculosis* by three-band MPCR was 200 organisms, whereas two-band MPCR could detect two organisms (data not shown). Thus three-band MPCR was 100 times less sensitive than the two-band MPCR.

When tested with standard species and strains of mycobacteria, the targets from IS6110 and IS1081 were amplified with all members of the *M. tuberculosis* complex; 162 bp DNA was amplified with all mycobacteria but none of the targets were amplified with non-mycobacterial organisms (Table 2). A non-specific, but consistent, amplification of the 200 bp DNA fragment in three-band MPCR was observed with ge-

Table 2 **Amplification of target DNA from mycobacterial and non-mycobacterial organisms in three-band multiplex polymerase chain reaction**

Organism	Amplification of the target DNA		
	162 bp	248 bp	375 bp
M. tuberculosis complex			
M. tuberculosis H37Ra, H37Rv	+	+	+
M. bovis, *M. bovis* BCG	+	+	+
M. africanum, *M. microti*	+	+	+
Non-tuberculous mycobacteria			
M. ulcerans, *M. kansasii*, *M. simiae*	+	–	–
M. marinum, *M. szulgai*, *M. gordonae*	+	–	–
M. asiaticum, *M. scrofulaceum*	+	–	–
M. flavescens, *M. xenopi*, *M. gastri*	+	–	–
M. *avium*[a], *M. intracellulare*[a]	+	–	–
M. malmoense, *M. diernhoferi*	+	–	–
M. haemophilum, *M. terrae*	+	–	–
M. nonchromogenicum, *M. triviale*	+	–	–
M. fortuitum, *M. chelonei*, *M. phlei*	+	–	–
M. smegmatis, *M. vaccae*, *M. chitae*	+	–	–
Non–mycobacterial organisms			
E. coli, *C. diphtheriae*	–	–	–
H. influenzae, *N. asteroides*	–	–	–
Group A streptococcus	–	–	–

[a] *A product of 200 bp DNA was also present*

Table 3 **Results of three-band multiplex polymerase chain reaction with the clinical isolates of mycobacteria**

Clinical isolates	Amplification of the target DNA			Culture results
	375 bp	248 bp	162 bp	
1	+	+	+	*M. tuberculosis* complex
2–3	–	–	+	NTM
4–36	+	+	+	*M. tuberculosis* complex
37	–	–	+	NTM
38	+	+	+	*M. tuberculosis* complex
39	–	–	+	NTM
40–43	+	+	+	*M. tuberculosis* complex
44–53	–	–	+	*M. avium*[a]

[a] *In addition to 162 bp DNA, a fragment of 200 bp DNA was amplified with these isolates*
NTM = non-tuberculous mycobacteria

nomic DNA from *M. avium-intracellulare* (MAI) complex (Table 2). Further experiments showed that the primer pair SA1-SA2 was responsible for amplification of the 200 bp DNA band (data not shown).

A total of 53 mycobacterial isolates from clinical specimens were tested to identify and differentiate *M. tuberculosis* complex from NTM. Of these, 39 isolates were *M. tuberculosis* and 14 were NTM; 10 NTM isolates were *M. avium* and 4 were mycobacteria other than *M. tuberculosis* and MAI complex. All the expected products of three-band and two-band MPCRs were amplified with *M. tuberculosis* isolates, whereas only the 162 bp DNA fragment was amplified with NTM (Table 3, data shown for three-band MPCR). A 200 bp DNA fragment was amplified from the genomic DNA of all *M. avium* isolates in three-band MPCR (Table 3). These results suggest that the three-band MPCR may also be useful in differentiating *M. tuberculosis* complex and MAI complex from other mycobacteria.

To test the applicability of MPCRs in the diagnosis of tuberculosis, three-band and two-band MPCR were evaluated for their diagnostic potential using clinical specimens from patients suspected of tuberculosis. Two-band and three-band MPCR were considered positive for *M. tuberculosis* when *M. tuberculosis*-specific DNA bands of the expected size were seen on the agarose gels. A specimen was considered to have NTM when only 162 bp DNA was amplified.

A total of 151 specimens were obtained from clinically suspected pulmonary (n = 64) and extra-pulmonary (n = 87) tuberculosis patients. Twenty-four (24) of these specimens were smear and/or culture positive and 127 were negative for smear and culture (Table 4). Among culture-positive specimens (n = 23), 22 specimens had *M. tuberculosis* and 1 specimen had NTM. All of the 127 smear- and/or culture-negative specimens were negative by both MPCRs, showing 100% specificity (Table 4). The sample containing NTM was positive only for 162 bp DNA by both MPCRs.

Table 4 **Results of multiplex polymerase chain reactions (MPCRs) with clinical samples from suspected tuberculosis patients**

Smear	Culture	No. of samples	Samples positive for			
			Two-band MPCR		Three-band MPCR	
			No.	%	No.	%
≥ 2+	+	5	5	100	5	100
1+	+	12	11[a]	92	9[a]	75
–	+	6	4	67	3	50
1+	–	1	1	100	1	100
–	–	127	0	0	0	0

[a]*One of these samples had non-tuberculous mycobacteria and it was positive only for 162 bp DNA.*
Compared to smear and/or culture results, two-band MPCR had a sensitivity of 88% and specificity of 100%; three-band MPCR had a sensitivity of 75% and specificity of 100%.

With regard to detection of *M. tuberculosis* in smear- and/or culture-positive specimens, two-band and three-band MPCRs were positive with all culture-positive specimens with a smear-positivity index of ≥ 2+ (Table 4). However, the positivity of both MPCRs decreased with culture-positive specimens with a smear-positivity index of 1+ or negative (Table 4). The decrease in MPCR positivity in culture-positive and smear-negative specimens was more pronounced with three-band MPCR than with two-band MPCR (Table 4). The overall sensitivity of two-band and three-band MPCRs with clinical specimens, compared to culture and/or smear results was 88% and 75%, respectively (Table 4).

Discussion

Although PCR techniques have been widely evaluated in the diagnosis of tuberculosis, the reports have mostly focused on the detection of *M. tuberculosis* [*1–6*]. However, the isolation of NTM from a large proportion of immunocompromised subjects [*7,8*] suggests that PCR methods that can differentiate between *M. tuberculosis* complex and NTM are needed. This requires the development of MPCRs using oligonucleotide primers capable of amplifying DNA targets, some of which should be specific for *M. tuberculosis* complex and others common to all mycobacteria. Of the multiple copy gene targets we selected to establish MPCRs in our study, the DNA targets in IS6110 and IS1081 are *M. tuberculosis* complex specific [*15–19*], whereas the 162 bp DNA in the gene encoding antigen 85 complex is common to all mycobacteria [*14*].

Several researchers have demonstrated that the use of IS6110 as a target for PCR amplification gives the best sensitivity and specificity in the diagnosis of tuberculosis [*2,3,5,20*]. However, a multicentre study has suggested that the sensitivity of different targets within the IS6110 sequence varies considerably [*21*]. Thus, there was a need to identify the most sensitive targets in the IS6110 sequence in order to establish highly sensitive MPCRs. Our results show that of the four different targets selected within the IS6110 sequence, the sensitivity of amplification decreased as the size of the amplified DNA increased, i.e. the amplifi-

cation of 123 bp and 245 bp DNA was most sensitive (detection limit = DNA from 0.2 organisms) followed by 375 bp DNA (detection limit = DNA from 2 organisms) and 580 bp DNA (detection limit = DNA from 20 organisms). Although the amplification of 123 bp DNA was as sensitive as 245 bp DNA, the 123 bp DNA was not targeted in the MPCRs because it could not be differentiated from the common mycobacterial target of 162 bp DNA by gel electrophoresis.

When tested with standard mycobacterial species and strains obtained from ATCC and mycobacterial cultures isolated from clinical specimens, the three-band and two-band MPCRs were able to distinguish *M. tuberculosis* complex from other mycobacteria. These observations suggest that MPCRs could be useful in the detection of NTM, which are mostly associated with immunodeficiency states, which are found in conditions such as AIDS. The amplification of a 200 bp DNA fragment from the *M. avium-intracellulare* (MAI) complex in three-band MPCR suggests that this test may also be useful in differentiating MAI complex from other NTM.

A total of 151 coded clinical samples were tested with the MPCR assays developed. Each sample was processed for smear, culture and two-band and three-band MPCRs. The results of two-band and three-band MPCR were positive with all culture-positive specimens with a smear-positivity index of ⊕ 2+. However, the MPCRs positivity decreased appreciably with smear-negative and culture-positive specimens. Very few researchers have used these types of samples, but those who have have reported similar PCR results [*22,23*]. The decrease in the MPCRs positivity with smear-negative and culture-positive specimens was more pronounced with three band MPCR than with two-band MPCR. Thus three-band MPCR was 100 times less sensitive than two-band MPCR using DNA isolated from culture grown *M. tuberculosis*.

In conclusion, our results demonstrate that two-band and three-band MPCRs differentiated between standard strains and clinical isolates of *M. tuberculosis* and NTM. Since three-band MPCR could also specifically detect MAI complex, it may be useful in the identification of different major groups of pathogenic mycobacteria in immunocompromised patients, e.g. AIDS patients. Because of the superior sensitivity of two-band MPCR, it could be used in the early and specific diagnosis of clinically suspected tuberculosis patients. However, for smear- and MPCR-negative specimens from clinically suspected tuberculosis patients, the diagnosis should be confirmed by culture.

Acknowledgements

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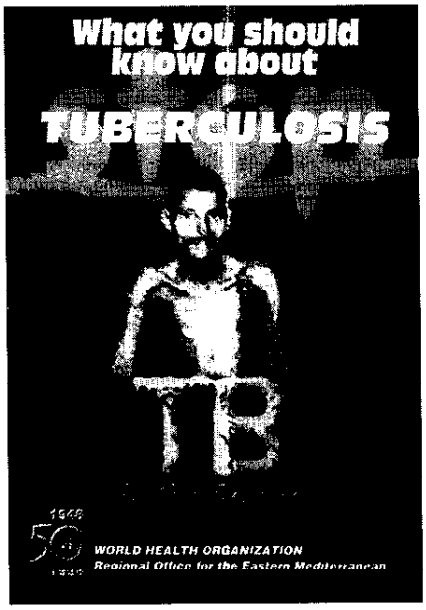

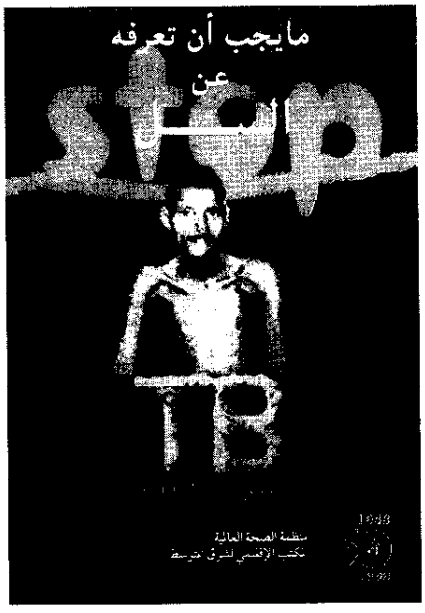

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