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Evidence of ‘amplifier effect’ in pulmonary multidrug-resistant tuberculosis: report of three cases[☆]

A. Umubyeyi^{a,b,*}, I.C. Shamputa^{a,c}, L. Rigouts^a, A. Dediste^b,
M. Struelens^d, F. Portaels^a

^a *Mycobacteriology Unit, Institute of Tropical Medicine, B-2000 Antwerp, Belgium*

^b *Service of Microbiology, CHU St Pierre, Brussels, Belgium*

^c *Microbiology Unit, Tropical Diseases Research Centre, PO Box 71769, Ndola, Zambia*

^d *Microbiology Department, Erasme Hospital, Brussels, Belgium*

Received 31 July 2006; received in revised form 6 November 2006; accepted 23 January 2007

Corresponding Editor: J. Peter Donnelly, Nijmegen, The Netherlands

KEYWORDS

Mycobacterium tuberculosis;
Multidrug-resistant tuberculosis (MDR TB);
Amplifier effect;
Spoligotyping,
MIRU-VNTR

Summary

Introduction: A cluster of three related cases of tuberculosis (TB) with primary multidrug resistance was investigated at the Centre Hospitalier Universitaire de Kigali (CHUK) in Rwanda. The patients were HIV-1/2 seronegative. Patients 1 and 2 were hospitalized in the same room of CHUK for one month. Patient 3 was a younger sibling of patient 2.

Methods: Drug susceptibility of two consecutive *Mycobacterium tuberculosis* isolates from each patient was tested by the BACTEC 460 radiometric method. DNA fingerprinting was performed using spoligotyping and mycobacterial interspersed repetitive units of variable numbers of tandem repeats (MIRU-VNTR) analysis. All patients initially received the World Health Organization category I regimen.

Results: The isolates collected during the first TB episode were resistant to isoniazid, rifampin and ethambutol. After subsequent retreatment regimens with rifampin, isoniazid, streptomycin, pyrazinamide (8 months) and rifampin, isoniazid, streptomycin, pyrazinamide, ciprofloxacin (21 months), patients 1 and 2 developed additional resistance to streptomycin and quinolones. Patient 3 received only the category I regimen and consecutive isolates retained the initial drug susceptibility pattern. All isolates were genetically indistinguishable by spoligotyping and MIRU-VNTR, indicating the same origin.

Conclusions: These observations highlight the risk of nosocomial transmission of multidrug-resistant (MDR) TB and the possible selection of secondary resistance to second-line drugs if a single new drug is added at the time of retreatment of MDR TB patients.

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[☆] Presented at the 36th Union Conference on Lung Health, Paris, France, 18–22 October 2005 (Abstract PS-1252-20).

* Corresponding author. Tel.: +32 3 247 6473; fax: +32 3 247 6333.

E-mail address: alainenyaruhirira@hotmail.com (A. Umubyeyi).

Introduction

Tuberculosis (TB) caused by bacteria belonging to the *Mycobacterium tuberculosis* complex, is one of the leading infectious diseases worldwide. People in contact with contagious TB patients are at risk of infection, and may even develop active disease faster when they are frequently in close contact with the contagious source because of the high bacillary load transmitted.^{1–3} The detection of sources of infection implies discrimination between strains of *M. tuberculosis*.^{4–6} The development of DNA-fingerprinting tools in the last two decades has considerably improved the capacity to distinguish *M. tuberculosis* strains, thereby enabling tracking of strains in the community, and has made the designing of prevention and control strategies to block further transmission possible.^{7–9} These tools include the insertion sequence IS6110-based restriction fragment length polymorphism (IS6110-RFLP), spoligotyping, and more recently, typing based on variable numbers of tandem repeats (VNTRs) of mycobacterial interspersed repetitive units (MIRU-VNTR).^{10–14} The latter methods have an advantage over IS6110-RFLP because they are less labor intensive, involve an amplification step that requires very little DNA, and can be easily interpreted.^{15–17}

Another phenomenon to be considered in the assessment of the clinical effectiveness of a treatment regimen in resistant TB is termed the 'amplifier effect of short-course chemotherapy'.¹⁸ This term describes the process by which patients infected with strains resistant to at least one drug not only fail short-course chemotherapy (SCC), but in the process may recruit additional resistance to other drugs.¹⁹

We report herein an example of a 'case-contact' transmission of TB using DNA fingerprinting analyses, and demonstrate the 'amplifier effect' of drug resistance in multidrug-resistant (MDR) TB retreatment patients.

Patient histories

Patient 1

A 23-year-old female was diagnosed with TB by smear microscopy (new case) in January 2001 at Gikondo Health Center

(Table 1). She was HIV-1/2 seronegative, had no family ties with patients 2 and 3, but was hospitalized for 1 month in the same room as patient 2 during the first treatment phase of category II (2(SERHZ)₇/1(ERHZ)₇/5(ERH)₃) (July 2001). She received the World Health Organization (WHO) category I treatment regimen (2(ERHZ)₇/4(RH)₃) but remained smear positive after 5 months after which the patient was retreated with a category II regimen (Table 2). In March 2002, the patient received the same retreatment regimen with the addition of ciprofloxacin.

Patient 2

This female, aged 22 years, was first hospitalized in July 2001 at Gikondo Health Center, in the same room as patient 2, for malaria (Table 1). She was later diagnosed with TB by smear microscopy (new case) in September 2002 at the Centre Hospitalier Universitaire of Kigali (CHUK) and was seronegative for HIV-1/2. She was transferred to Gikondo Health Center where she begun treatment with the category I regimen. However, the patient remained smear positive after 5 months of treatment, and was started on the category II regimen in March 2003; she was hospitalized several times (Table 2). Three months after starting the category II treatment (June 2003), the patient was confirmed to be an MDR case, with the isolate being resistant to isoniazid (H), rifampin (R), ethambutol (E) and rifabutin. She began another retreatment with streptomycin (S), isoniazid, ofloxacin and clofazimine, but this patient remained smear positive in November, and her treatment was consequently changed to second-line drugs (clofazimine, ofloxacin, prothionamide and pyrazinamide). However, the patient died four months later.

Patient 3

Patient 3 was a younger sibling of patient 2. She was 16 years old and seronegative for HIV-1/2 (Table 1). She took care of her sister while in hospital and later accompanied her to receive treatment from a DOT center. In September 2003, she was diagnosed with TB (new case) and begun treatment with the category I (2(ERH)₇/4(RH)₃)

Table 1 Patient characteristics and DNA fingerprinting of different isolates

Patient	Age/sex	HIV test	TB history	Diagnosed with TB	Hospitalization period (reason)	Date of sputum collection (mo/y)	Isolate No.	MIRU-VNTR	Spoligotype profile
1	23/F	Negative	No	01/2001	7/2001 (TB disease)	02/2001	A1	223325153423	77777777760731
						10/2003	B1	223325153423	77777777760731
2	22/F	Negative	No	09/2002	7/2001 (malaria)	09/2002	A2	223325153423	77777777760731
						11/2003	B2	223325153423	77777777760731
3	16/F	Negative	No*	09/2003	7/2001 (care of sister)	09/2003	A3	223325153423	77777777760731
						02/2004	B3	223325153423	77777777760731

F, female; MIRU-VNTR, mycobacterial interspersed repetitive units of variable numbers of tandem repeats.

(Spoligotype data shown are presented in binary format recently proposed by Filliol I, et al. *Emerg Infect Dis* 2002;11:1347–9.)

*Case contact: Yes.

Table 2 Chronological treatment outcome and DST results of three patients

Patient No.	DST result of isolates A	Treatment I Cat I (5 month)	Outcome	Retreatment Cat I or Cat II + Cpx/Ofx	Outcome	DST result for isolates B	Retreatment outcome
1	HRE-(r) + Ofx-(s)	HREZ	Failure	HREZS (8 months) HREZS + Cpx (21 months)	Failure	HRES/Cpx + Ofx-(r)	MDR ongoing
2	HRE-(r) + Ofx-(s)	HREZ	Failure	HREZS (3 months) HS + Ofx + Cfz (21months)	Failure	HRES/Cpx + Ofx-(r)	Died after 4 months
3	HRE-(r) + Ofx-(s)	HREZ	Failure	HREZ	Failure	HRE-(r) + Ofx-(s)	MDR ongoing

(s), susceptible; (r), resistant; H, isoniazid; R, rifampin; E, ethambutol; Z, pyrazinamide; S, streptomycin; Cpx, ciprofloxacin; Ofx, ofloxacin; Cfz, clofazimine.

regimen. However, the patient remained smear positive after three months of treatment. The *M. tuberculosis* isolate cultured from this patient was found to be resistant to isoniazid, rifampin, ethambutol and rifabutin like the one initially cultured from her older sister after the first regimen. In April 2004, she was transferred to France where she is still undergoing treatment with second-line drugs.

Methods

Samples and cultures

A sputum sample was collected for culture from each patient before or within the first two weeks of treatment during the first and second TB episodes. The samples were mixed with 1% cetylpyridinium chloride (CPC) and shipped to the national reference laboratory (LRN) of Kigali for analysis. Upon receipt at the LRN, each sample was cultured on Löwenstein–Jensen medium and Coletsos after decontamination using the Petroff procedure.²⁰ Primary cultures that resembled *M. tuberculosis* were sent to the Mycobacteriology Unit at the Saint Pierre Hospital laboratory, Brussels for species identification and drug susceptibility testing (DST), and to the Mycobacteriology Unit, Institute of Tropical Medicine, Antwerp for fingerprinting. Primary cultures were identified by classical methods and DST was performed with rifampin (2 µg/ml), isoniazid (0.2 µg/ml), streptomycin (4 µg/ml) and ethambutol (5 µg/ml) using the BACTEC 460 radiometric method as described by Siddiqi et al.^{21–23}

Phenotypic susceptibility testing for pyrazinamide was not performed, because the results of this test can be difficult to reproduce and may not correlate well with drug susceptibility in vivo.^{24,25} All MDR strains were further subjected to second-line antituberculosis drug testing at the Saint Pierre Hospital laboratory, Brussels. The drugs tested included ethionamide (20 µg/ml), ofloxacin (2 µg/ml), ciprofloxacin (2 µg/ml), amikacin (10 µg/ml), kanamycin (20 µg/ml) and rifabutin (1 µg/ml).^{22,23}

Quality control was established by comparing the results of DST performed at Saint Pierre and the Pasteur Institute, Paris, and these were 100% concordant. This study was approved by the Ethics Review Board at the Centre Hospitalier Universitaire de Kigali (CHUK).

HIV testing

The HIV status was tested using two enzyme-linked immunosorbent assays (ELISA): Vironostika HIV Uni-form II (bioMérieux, Marcy l'Étoile, France) and Murex HIV 1.2.0 (Abbott Murex, IL, USA).

DNA extraction

The genomic DNA used for PCR analysis was obtained by resuspending mycobacterial colonies into 200 µl in 1× TE (10 mM Tris-HCl, 1 mM EDTA (pH 8.0)) followed by boiling for 5 min in a water bath.

Spoligotyping

Spoligotyping was performed with a commercial kit (Isogen Bioscience BV, Maarssen, The Netherlands) by the previously described method.¹⁰ Briefly, 4 µl of the heat-killed bacterial suspension from each sample was used for amplification of the direct-repeat (DR) region with oligonucleotides DRa (5' biotinylated) and DRb. The labeled amplicons were used as probes for hybridization with a set of 43 known oligonucleotide spacer sequences of *M. tuberculosis* H37Rv and *M. bovis* BCG P3 covalently bound to a nylon membrane (Amersham Biosciences, Little Chalfont, UK). Bound PCR fragments were detected with a streptavidin horseradish peroxidase-enhanced conjugate and an enhanced chemiluminescence system, followed by exposure to ECL hyperfilm (Amersham Pharmacia-Biotech, Roosendaal, The Netherlands). The spoligotyping patterns were compared using Excel computer software.

MIRU-VNTR analysis

PCRs were carried out by using the Hot Start Taq DNA polymerase kit (Qiagen, Hilden, Germany), as described elsewhere.¹² The PCR fragments were analyzed by agarose gel electrophoresis using 3% NuSieve agarose (Cambrex Biosciences, USA). The sizes of the amplicons were estimated using a Gene Ruler™ 100-bp ladder (MBI Fermentas).

Results and discussion

All initial *M. tuberculosis* isolates collected from the three patients had identical fingerprints by spoligotyping and

MIRU-VNTR analysis (Table 1), thereby suggesting that the patients shared a common source of infection. Given the fact that none of these patients had a past history of TB, or cases of active TB in their direct environment, and that patients 1 and 2 shared the same room for more than one month during the infective stage of patient 1 (smear positive), it is most likely that the MDR *M. tuberculosis* strain was transmitted in the hospital from patient 1 to patient 2. From the data we obtained, the initial source of infection for patient 1 is probably the mother who died of TB/HIV co-infection two years earlier. It is not clear whether patient 3 was infected by patient 1 while taking care of her sister (July 2001) or later by her sister when she developed active TB (September 2002). The fact that initial and subsequent *M. tuberculosis* isolates showed identical fingerprints clearly demonstrates treatment failure in all three cases.

None of the patients hospitalized in the same room with patients 1 and 2 developed TB. In addition, no other family member developed TB except for the younger sister of patient 2 who took care of her while in hospital.

During the first phase of treatment, the three patients' isolates had the same MDR profile to the first-line drugs tested and were all susceptible to streptomycin and the second-line drugs, ofloxacin and kanamycin (Table 2). Although we do not have intermediate *M. tuberculosis* isolates after retreatment with category II, and the resistance profile to pyrazinamide was not determined, the final drug-resistance profiles suggest that patients 1 and 2 probably independently developed resistance to streptomycin during retreatment with category II, since in fact streptomycin was added as a single new drug to the four drugs, for which resistance already existed to at least three (isoniazid, rifampin, ethambutol). After receiving 8 months of category II retreatment, and meanwhile probable acquisition of resistance to streptomycin, ciprofloxacin was added for retreatment of patient 1 and therefore de facto could be considered as monotherapy. The strain finally became resistant to ciprofloxacin and ofloxacin (Table 2). Cross-resistance between fluoroquinolones is a well-known phenomenon.³ On the other hand, for patient 2, ofloxacin and clofazimine were added to streptomycin and isoniazid after three months of the category II treatment regimen that failed. The strain developed resistance to ofloxacin and ciprofloxacin, but remained susceptible to clofazimine. This drug has been used for leprosy and has excellent in vitro inhibitory activity against *M. tuberculosis*, but there is little or no information on its in vivo activity.^{26,27} It was demonstrated in vitro that clofazimine in combination with isoniazid may result in synergistic activity against *M. tuberculosis*.²⁸ This synergistic activity, however, may be more pronounced against the wild-type *M. tuberculosis* strain than against strains harboring the *katG* S315T mutation.²⁸ Since our isolate was already resistant to isoniazid, it is likely that clofazimine had no synergistic activity on the isolate. The additional resistance to streptomycin results from the 'amplifier effect'.^{29,30}

This phenomenon in which additional resistance to anti-tuberculosis drugs is observed among polyresistant patients who receive the standard SCC regimen has previously been described, but its frequency is not well known.²⁹

In a recent study, Quy et al. showed the limitations of the (2(SERHZ)₇/6(HE)₇) in preventing the amplification effect in patients with primary resistance even other than MDR TB,

including single drug resistance. In their report the authors suggest that replacing streptomycin in the standard regimen and/or adding a third drug to the continuation phase may be one possible way of breaking the amplification juggernaut.^{30,31}

The acquired resistance to the quinolones clearly showed that second-line drugs should never be administered as a single new drug, but rather be part of a complete MDR TB treatment regimen including at least three drugs that have not been used before.³⁰

These alarming findings on the prevalence of resistance to second-line drugs, especially among MDR TB patients, highlight the risk of producing primary resistance to fluoroquinolones, creating incurable TB strains and jeopardizing the potential of fluoroquinolones to become part of first-line anti-TB drug therapy in directly observed treatment short-course (DOTS)-plus. The present study confirms that individuals who are in frequent contact with contagious TB patients have a high risk of contracting TB and progressing to active disease. Nosocomial transmission of *M. tuberculosis* in treatment failures constitutes a real risk. Although the sample size of our data is small to draw definitive conclusions, our report nonetheless indicates the potential risk of producing resistance to additional drugs if patients infected with polyresistant *M. tuberculosis* strains receive the standard SCC and when a single drug (e.g. a quinolone) is added to a failed treatment regime, confirming the transmission of MDR strains in three cases.

Acknowledgments

We thank the National TB program and the CGRI (Commissariat Général aux Relations Internationales) for financial support. This study was also partially supported by the Damien Foundation (Brussels, Belgium) and the Fund for Scientific Research of Flanders (Brussels, Belgium; FWO grant No. G.0471.03N). ICS was supported by a grant from Ackerman & Van Haaren NV. We thank the staff of the Institut Pasteur of Paris (Centre National de Référence des Mycobactéries), the Mycobacteriology Unit of the Institute of Tropical Medicine (Antwerp) and the National Laboratory of Kigali for excellent technical assistance.

Conflict of interest: No conflict of interest to declare.

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