JIRESH-Biotech

Journal of Innovation and Research in Health Sciences & Biotechnology: Open Access

Volume 1, Issue 1, September 2015 http://jiresh-biotech.edmgr.com

Original Article

Infection with Multiple Strains of *Mycobacterium tuberculosis* in Patients with Culture Confirmed Pulmonary Tuberculosis in the KwaZulu-Natal Population

Prashini Moodley[1], Nabihah Tayob[2], Saajida Mahomed[1] and A. Willem Sturm[1]

[1]Department of Infection Prevention and Control, Nelson R Mandela School of Medicine, University of KwaZulu-Natal and [2]Biostatics Unit, Medical Research Council, Durban, South Africa

Corresponding Author: Prashini Moodley, MBChB, MMed, PhD; (t): +27-312604395; E-mail: moodleyp@ukzn.ac.za

J. Innov. Res. Health Sc. Biotech. 2015; 1(1): 44 – 48. doi: 10.18644/jiresh-biotech.0000007

ABSTRACT

Background: There is accumulating evidence that patients with tuberculosis could very well be infected with multiple strains of *Mycobacterium tuberculosis*. This may be the result of clonal heterogeneity or of super-infection with one or more new strains. **Methods:** All the culture proved cases of pulmonary tuberculosis from patients in KwaZulu-Natal, diagnosed between 1 January 2006 and 30 June 2007 were included in the study. Patients from whom two or more specimens were sent on the same day for culture of *M. tuberculosis* between 1 January 2006 and 30 June 2007 were identified. Differences in the susceptibility profile were used to determine the diversity between isolates. **Results:** During the study period, 2617 patients had multiple specimens collected on the same day. Of these, 166 (6.3%) displayed discordant susceptibility profiles. From 56 (33.7%) of these 166 patients, an MDR isolate was grown from one of the specimens, from 13 (7.8%) a pre-XDR isolate, from 10 (6.0%) an XDR isolate. **Conclusion:** Patients with pulmonary tuberculosis may be infected with multiple strains of different resistance profiles. Drug-resistant tuberculosis may be under-diagnosed, and clinicians should consider sending more than one specimen for culture and susceptibility testing, particularly in patients who are not responding to treatments.

Keywords: *multiple strain infection, pulmonary tuberculosis, population.*

RÉSUMÉ

Contexte: Plusieurs preuves suggèrent que les patients infectés par la tuberculose peuvent être infectés par des souches multiples de *Mycobacterium tuberculosis*. Ceci pourrait être le résultat d'une hétérogénéité clonale ou d'une super-infection avec une ou plusieurs souches. **Méthodologie :** Tous les cas de cultures avérés de tuberculose pulmonaire de patients à KwaZulu-Natal, diagnostiqués entre le 1er janvier 2006 et le 30 juin 2007, ont fait l'objet de cette étude. Les patients dont deux spécimens ou plus ont été envoyés le même jour pour culture de *M. tuberculosis* entre le 1er janvier 2006 et le 20 juin 2007 ont été identifiés. Les différences entre les profils de sensibilités ont été utilisées afin de déterminer la diversité entre les isolats. **Résultats :** Au cours de la période à l'étude, 2617 patients ont eu des spécimens multiples recueillis le même jour. Parmi ceux-ci, 166 (6.3%) ont montré des profils de sensibilités discordants. De ces 166 patients, 56 (33.7%) présentaient un isolat MDR cultivé à partir de l'un des spécimens, 13 (7.8%) présentaient un isolat pré-XDR et 10(6.0%) présentaient un isolat XDR. **Conclusion :** Les patients ayant une tuberculose pulmonaire peuvent être infectés par des souches multiples avec des profils différents de résistance. Une tuberculose résistante aux médicaments peut être sous-diagnostiquée, et les cliniciens devraient considérer envoyer plus d'un spécimen pour culture et pour test de sensibilité, particulièrement pour les patients qui ne répondent pas au traitement.

Mots clés : *infection due aux souches multiples, tuberculose pulmonaire, population.*

Submitted 12/07/2015, accepted 21/09/2015 http://jiresh-biotech.edmgr.com

INTRODUCTION

Since the 1960s, the general concept has been that most cases of the tuberculosis resulted from the reactivation of latent infections that had a subclinical course at the time of acquisition (1). This concept has been challenged during the last decade with the introduction of genotyping methods which showed that patients with latent infection do acquire new exogenous infections with another strain (2). It also became evident that patients may be infected with more than one strain at the same time (3-6). Infections with multiple strains can be the result of clonal

heterogeneity or of super-infection with one or more new strains. The basis of clonal heterogeneity is the occurrence of mutations during infection, which results in multiple strains in one patient derived from a common ancestor. Super infection on the other hand, results in infection with multiple strains from a different origin.

Independent of the mechanism through which infections with multiple strains occur, these strains can differ in resistance pattern as well as in virulence. In patients, on treatment, differences in the resistance pattern, resulted in the dominance of the strain resistant to the drugs used. The difference in virulence can result in the dominance of the most virulent strain as the disease progresses.

While the number of publications on multiple infections increases, no study has been published that investigated the frequency of its occurrence at the population level. We report the prevalence of multiple strain infection in culture confirmed cases of pulmonary tuberculosis in the KwaZulu-Natal province of South Africa.

MATERIALS AND METHODS

All culture proven cases of pulmonary tuberculosis from patients in KwaZulu-Natal, diagnosed between 1 January 2006 and 30 June 2007 were included in the study. As per the South African National TB Treatment Guidelines (7), patients suspected of TB are meant to provide two sputum specimens on consecutive days. This practice is difficult to monitor, and patients are often given the sputum specimen containers and provide a specimen at their convenience. In this study, patients were identified from whom two or more specimens were sent on the same day for the culture of *Mycobacterium tuberculosis*. Differences in the susceptibility profile were then used to determine the diversity between isolates.

Sputa were digested and decontaminated using the NALC-NaOH method (8). Cultures were performed using the MGIT automated system (Becton and Dickinson Microbiology Systems, Maryland, USA). Positive readings were checked for acid-fast bacilli by means of the Auramine stain. This was followed by subculture for identification tests and susceptibility testing directly out of the MGiT broth. The 1 % proportional method on Middlebrook 7H10 agar plates was employed (9). Susceptibility test results were available for isoniazid (1 mg/L), rifampicin (1 mg/L), ethambutol (5 mg/L), streptomycin (2 mg/L), kanamycin (5mg/L) and ofloxacin (1 mg/L). Multi-drug resistance (MDR) is defined as resistance to at least isoniazid and rifampicin, pre-XDR as an MDR resistance pattern with additional resistance against either ofloxacin or kanamycin and extensive drug resistance (XDR) as an MDR pattern with resistance to ofloxacin and kanamycin. The term "poly-drug resistance" was used for organisms resistant to 2 or more drugs without identifying specific combinations. Poly-drug resistance, therefore, includes MDR, pre-XDR and XDR.

RESULTS

During the study period specimens for the laboratory diagnosis of tuberculosis were received from 20858 patients. Multiple specimens were received from 3511 (16.8 %) of these patients. This includes 2617 (12.5 %) patients with multiple specimens collected on the same day. An MDR isolate was grown from at least one of 603 (23.0%) of these specimens, a pre-XDR isolate was grown from 21 (0.8%), and an XDR isolate from 112 (4.3%).

Of those with multiple specimens on the same day, 166 (6.3%) displayed discordant susceptibility profiles. Fifty-six (33.7%) of these 166 patients were discordant with respect to their MDR status, 13 (7.8%) with respect to pre-XDR status and 10 (6.0%) with respect to XDR status.

Fifty-one (30.7%) patients with a fully susceptible isolate in one specimen had an isolate resistant to one or more drugs in the second specimen collected on the same day (**Table 1**). Twenty-two (13.3%) patients had a single drug resistant isolate in one specimen and a poly-drug resistant isolate in the second specimen (**Table 2**) while 92 (55.4%) had a poly-drug resistant isolate in one specimen and a more resistant poly-drug resistant one in the other specimen (**Table 3**).

DISCUSSION

We report on mixed infections in 6.3 % of patients with pulmonary tuberculosis in the province of KwaZulu-Natal, South Africa. This study is unique in that it reports on all culture confirmed cases over an 18 month period in a province with a population of over 9 million (10) Another study from KwaZulu-Natal that looked at mixed infections in patients that died from tuberculosis reported a prevalence of 9% (11). A study from a different part of South Africa found 23 % of 48 patients with MDR tuberculosis to be infected with more than one strain (3). In contrast, a study from Malawi reported mixed infection in 2.8 % of 160 patients with culture proven pulmonary tuberculosis (4). Two recent studies from Taiwan reported mixed infections in 3% and 11.5 % of patients, respectively (5, 6). While other studies used molecular techniques to establish the presence of mixed infections in one specimen, our results are based on phenotypic differences in isolates from different specimens from the same patient collected on the same day.

The 1% proportional method of drug susceptibility testing allows 1 % of the bacterial population to be resistant against the drug tested while the predominant population is susceptible. This is based on the reported high mutation frequency in genes coding for the drug targets.

Table 1. Resistance patterns of *M.tuberculosis* isolates from patients with a fully susceptible isolate from a second specimen collected on the same day

Resistant to:	No. of patients
isoniazid	14
rifampicin	9
streptomycin	8
ethambutol	1
isoniazid + Streptomycin	4
isoniazid + rifampicin (MDR)	4
isoniazid + rifampicin + streptomycin (MDR)	10
isoniazid + rifampicin + ethambuthol (MDR)	1
isoniazid + rifampicin + streptomycin + ethambutol (M	1
isoniazid + rifampicin + streptomycin + Kanamycin (pre	1
isoniazid + rifampicin + Kanamycin + ofloxacin (XDR)	1
Total no. of patients	54

Table 2. Resistance patterns of isolates of patients with a single drug resistant isolate (A) in one and a multiple drug resistant isolate (B) in a second specimen collected on the same day

Isolate A resistant to :	Isolate B resistant to:	No. of patients
isoniazid (n=15)	isoniazid + streptomycin	4
	isoniazid + rifampicin (MDR)	5
	isoniazid + rifampicin + streptomycin (MDR)	2
	isoniazid + rifampicin + ethambutol (MDR)	1
	isoniazid + rifampicin + streptomycin + ethambutol (MDR)	1
	isoniazid + rifampicin + kanamycin (MDR)	1
	isoniazid + rifampicin + kanamycin + ofloxacin + streptomycin + ethamb	1
rifampicin (n=7)	rifampicin + streptomycin	1
	isoniazid + rifampicin (MDR)	5
	isoniazid + rifampicin + streptomycin (MDR)	1
streptomycin (n=3)	isoniazid + streptomycin	1
	isoniazid + rifampicin + streptomycin (MDR)	2
	Total no. of patients	25

Table 3. Resistance patterns of isolates of patients with different patterns of poly-drug resistance in isolates grown from two specimens collected on the same day

Isolate A resistant to:	Isolate B resistant to:	No. of patients
isoniazid + streptomycin (n=12)	isoniazid + rifampicin (MDR)	2
	isoniazid + rifampicin + streptomycin (MDR)	8
	isoniazid + rifampicin + streptomycin + ethambutol (MDR)	2
rifampicin + streptomycin (n=1)	isoniazid + rifampicin + streptomycin (MDR)	1
isoniazid + rifampicin (MDR) (n=24)	isoniazid + rifampicin + streptomycin (MDR)	21
	isoniazid + rifampicin + streptomycin +ethambutol (MDR)	1
	isoniazid + rifampicin + kanamycin + ofloxacin (XDR)	1
	isoniazid + rifampicin + kanamycin + ofloxacin + streptomycin + ethambutol (XDR)	1
isoniazid + rifampicin + streptomycin (MDR) (n= 43)	isoniazid + rifampicin + kanamycin + streptomycin	4
	isoniazid + rifampicin + streptomycin + ethambutol	32
	isoniazid + rifampicin + kanamycin + streptomycin + ethambutol	3
	isoniazid + rifampicin + ofloxacin + streptomycin	1
	isoniazid + rifampicin + ofloxacin + streptomycin + ethambutol	1
	isoniazid + rifampicin + kanamycin + ofloxacin	1
	isoniazid + rifampicin + kanamycin + ofloxacin + streptomycin + ethambutol (XDR)	1
isoniazid + rifampicin + kanamycin (pre-XDR) (n=1)	isoniazid + rifampicin + kanamycin + ofloxacin + Streptomycin + ethambutol (XDR)	1
isoniazid + rifampicin + ofloxacin (n=1)	isoniazid + rifampicin + ofloxacin + ethambutol	1
isoniazid + rifampicin + kanamycin + ofloxacin (n=5)	isoniazid + rifampicin + kanamycin + ofloxacin + streptomycin	1
	isoniazid + rifampicin + kanamycin + ofloxacin + streptomycin + ethambutol (XDR)	4
isoniazid + rifampicin + kanamycin + ofloxacin + streptomycin (n=7)	isoniazid + rifampicin + ofloxacin + streptomycin + ethambutol	1
	isoniazid + rifampicin + kanamycin + ofloxacin + streptomycin + ethambutol (XDR)	6
isoniazid + rifampicin + Kanamycin + streptomycin + ethambutol (n=1)	isoniazid + rifampicin + kanamycin + ofloxacin + streptomycin + ethambutol (XDR)	1
isoniazid + kanamycin + ofloxacin + streptomycin + ethambutol (n=1)	isoniazid + rifampicin + kanamycin + ofloxacin + streptomycin + ethambutol (XDR)	1
	Total no. of patients	**96**

Treatment with a combination of drugs is supposed to prevent treatment failures due to a minority of resistant cells. Our findings suggest that the proportion of bacterial cells resistant to an antimicrobial agent varies between specimens, even when collected on the same day.

It is unlikely that our findings could be explained by the poor reproducibility of the phenotypic susceptibility testing, instead of the infection with multiple strains. The sensitivity of the drug susceptibility method used is high: 98.7% for isoniazid and 97.2% for rifampicin (12). Ethambutol has the lowest sensitivity of 89.3% (12). A difference in resistance for a specific drug only or in combination with others was found only in 59 of the 166 cases. In addition, the laboratory participates in a quality assurance program and performs well.

Only 16.8% of cases adhered to the National TB guidelines, by sending at least two specimens for laboratory diagnosis of tuberculosis. We argue that such differences must reflect infection with multiple strains as opposed to mutational events. In support of this is the fact that the multiple specimens from one individual were collected within one working day while the generation time of *M. tuberculosis* in the lungs of mice are in the order of 24 to 30 hours (13). It is unlikely that this will be much different in the lungs of man. Single bacterial cells with a mutation in a resistance gene that occurred on the day of specimen collection would not be discovered amidst the large population of non-mutated cells.

The reasons for multiple specimens sent in one day were not investigated, but is likely due to patient factors including clinical presentation. Patients who were producing more sputum, and possibly not responding to treatment may have been more likely to provide multiple specimens on one day. An indication that this might be the reason is that the percentage of MDR (23%) and XDR

(4.3%) is higher than the recently reported figures of 20% and 2% respectively for the whole province (14).

If drug susceptibility tests are performed from cultures grown on solid media, differences in resistance patterns between growths from multiple specimens can be the result of harvesting of different colonies from a mix. This cannot explain the difference in resistance patterns between isolates from multiple specimens in this study in which the susceptibility tests were performed directly from cultures grown in liquid media. In this situation, the cells of different strains, present in the broth were sub-cultured onto the susceptibility test agar. If both strains were present in all specimens, the resistance patterns would have been the same.

To explain our observations of isolates with different resistance patterns from different specimens collected on the same day, we postulate that there is an uneven distribution of the strains in the lungs of the patients with different specimens representing different parts of the lung. This needs further investigation.

Differentiation between the clonal heterogeneity and the super infection cannot be done without genotyping. Our study did not allow for that. However, our results support reports from others that patients with pulmonary tuberculosis can carry multiple strains. We further provide data on the magnitude of this phenomenon at the population level.

If we extrapolate our 6.3% prevalence of multiple strain infection to all patients with a culture confirmed diagnosis from whom only one specimen was received, approximately 1149 patients with multiple strain infection were missed. If the single specimen contained the most susceptible strain such patients were treated with inappropriate drugs till lack of response to treatment initiated a new culture request. This delay in diagnosis of the more resistant strains contributes to further spread of resistance.

If on the other hand, the single culture contained the most resistant strain, the patients were treated with the less effective and more toxic second or third line drugs. An important unanswered question is how much such patients would have benefitted from a combination of 1st and 2nd or 3rd line treatment.

These findings emphasise the complexity of, and challenges in the management of the TB epidemic in KwaZulu-Natal, and highlight the need for a molecular-based drug susceptibility test to avoid the -diagnosis of drug resistance.

Competing Interest:

Authors declare that they have no competing interest.

REFERENCES

1. Stead WW. Pathogenesis of a first episode of chronic pulmonary tuberculosis in man: recrudescence of residuals of the primary infection or exogenous reinfection? Am Rev Respir Dis 1967;95:729-45.

2. Crampin AC, Mwaungulu JN, Mwaungulu FD, Mwafulirwa DT, Munthali K, Floyd S, et al. Recurrent TB: relapse or reinfection? The effect of HIV in a general population cohort in Malawi. AIDS 2010. 2010;24:417-26.

3. Van Rie A, Victor TC, Richardson M, Johnson R, Van der Spuy GD, Murray EJ, et al. Reinfection and mixed infection cause changing Mycobacterium tuberculosis drug-resistance patterns. Am J Respir Crit Care Med. 2005;172:636-42.

4. Mallard K, McNerney R, Crampin AC, . Molecular detection of mixed infections of Mycobacterium tuberculosis strains in sputum samples from patients in Karonga District,. J Clin Microbiol. 2010;48:4512-8.

5. Wang JY, Hsu HL, Yu MC. The TAMI Group. Mixed infection with Beijing and non-Beijing strains in pulmonary tuberculosis in Taiwan: prevalence, risk factors, and dominant strain. Clin Microbiol Infect. 2010:1469-0691.

6. Huang HY, Tsai YS, Lee JJ, Chiang MC, Chen YH, Chiang CY, et al. Mixed Infection with Beijing and Non-Beijing Strains and Drug Resistance Pattern of Mycobacterium tuberculosis. J Clin Microbiol. 2010;48:4474-80.

7. Health. SANDo. National Tuberculosis Guidelines. National Tuberculosis Guidelines. 2007.

8. Lee L, V., Della-Latta P. Susceptibility tests by modified agar proportion. HD Isenberg, ed Clinical Microbiology Procedures Handbook AMS.2(7):7.

9. Delta-Latta P. Digestion-Decontamination Procedures. HD Isenberg, ed Clinical Microbiology Procedures Handbook AMS. 2004;2(7):1-2.

10. Africa. SS. Census 2001: Census in Brief. Pretoria;. Census 2001: Census in Brief Pretoria;. 2003.

11. Cohen T, Wilson D, Wallengren K. Mixed-strain Mycobacterium tuberculosis infections among patients dying in a hospital in KwaZulu-Natal, South Africa. J Clin Microbiol. 2011;49:385-8.

12. WHO. Anti-tuberculosis Drug Resistance in the World: Third Global Report. World Health Organisation. 2004.

13. Manca C, Tsenova L, Barry CEr. Mycobacterium tuberculosis CDC1551 induces a more vigorous host response in vivo and in vitro, but is not more virulent than other clinical isolates. J Immunol. 1999;162:6740-6.

14. Moodley P, Shah NS, Tayob N. Spread of Extensively Drug-Resistant Tuberculosis in KwaZulu-Natal Province, South Africa. PLoS One. 2011;6(5):17513.