Cytotoxicity Induction in A549 Alveolar Epithelial Cells by *Mycobacterium tuberculosis* Isolates Cultured in the Presence and Absence of Oxygen

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Abstract: *Background*: The emergence of drug resistant *Mycobacterium tuberculosis* and studies on clinical isolates of drug resistant *M. tuberculosis* highlight the need to obtain additional valuable information on this organism. In this study, we report on *M. tuberculosis* clinical isolates and laboratory strain induced cytotoxicity in A549 alveolar epithelial cells *in vitro*.

A549 cells were infected with 15 clinical isolates (members of the Beijing and F15/LAM4/KZN families, as well as isolates with unique DNA fingerprint), and two laboratory strains of *M. tuberculosis* grown in the presence of oxygen, and absence of oxygen. Lactate dehydrogenase released from A549 cells was quantified after infection to evaluate necrosis.

Results: The isolates grown in the absence of oxygen had a higher level of cytotoxicity than those grown in the presence of oxygen. Cytotoxicity levels induced by F15/LAM4/KZN and Beijing isolates grown in the absence of oxygen ranged from 18.4%-35.7% and 17.7%-27.5% respectively. The highest cytotoxicity level (35.7%) was produced by an extensively drug resistant isolate that belonged to the F15/LAM4/KZN family. Growth in the presence of oxygen resulted in lower levels of cytotoxicity, ranging from 14.3%-22.4% by the F15/LAM4/KZN isolates, and 13%-23.3% by the Beijing isolates. Isolates with unique DNA fingerprint induced cytotoxicity levels between 3.5%-8.5% (oxygen) and 5.3%-10.7% (no oxygen). The H37Rv and H37Ra bacilli induced cytotoxicity levels of 3.9% and 2.9% (oxygen) respectively and 5.7% and 3.3% (no oxygen) respectively.

Conclusion: These results correlate well with our previous findings on the adhesion and invasion rates of these isolates. The increased tissue destruction induced by the F15/LAM4/KZN isolate compared to the other isolates attest to its virulence and may partly explain the high mortality rates of patients infected with this strain in Kwa Zulu-Natal in 2005.

Keywords: Beijing, F15/LAM4/KZN, virulence, necrosis, drug-resistant, *in-vitro*.

BACKGROUND

South Africa is responsible for almost half of the total tuberculosis (TB) caseload among the eleven countries of the Southern Africa sub-region. It currently has the highest TB burden in the whole of Africa, and is placed 3rd among the twenty-two high TB burden countries in the world [1]. Historical neglect and poor management systems are contributing factors to the South African TB crisis. The progression of clinical isolates of *Mycobacterium tuberculosis* into multi drug and extensively drug resistant (MDR and XDR) isolates as well as the high rate of HIV-TB co-infection also contribute to the high ranking of South Africa within the global TB epidemic [2].

Understanding of the pathogenesis of TB can assist in the prevention of spread of the infection as well as the development of new vaccines and drugs. For infection to occur there must be an interaction between the host cell and the microbial pathogen. *M.*

tuberculosis bacilli are thought to enter its host as droplet nuclei *via* the nasopharynx and the bronchial tree to reach the alveolar space [3, 4]. Here, the bacilli are believed to make contact with the alveolar epithelium and colonise the alveolar tissue by adhering to and invading the epithelial cells. Previous reports show that *M. tuberculosis* bacilli adhere to and invade alveolar epithelial cells *in vitro* [5-8]. It has been suggested that this colonisation is followed by the destruction of the alveolar epithelium, granuloma formation and subsequent caseation and liquefaction of the granulomatous tissue [9].

Clarification of the virulence mechanism used by *M. tuberculosis* and the relationship between different virulence attributes will help in understanding the pathogenesis of TB. McDonough and Kress [5] reported that the virulent laboratory strain H37Rv was toxic to both epithelial monolayers and macrophages. Their results were confirmed by Dobo *et al.* [10], who also reported that this cytotoxicity was caused by cellular necrosis. These studies were performed in the presence of oxygen. However, the low redox potential in granuloma is likely to prevent aerobic metabolism [11]. It is therefore prudent to investigate the toxic effect of *M. tuberculosis* under anaerobic

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circumstances. There is also accumulating evidence that there are differences in virulence between different strains. Therefore, comparing successfully spreading strains with those that are less successful will result in additional valuable information.

In this study, we used clinical isolates of *M. tuberculosis* previously reported to adhere to and invade alveolar epithelial cells [7, 8] to investigate whether clinical isolates of *M. tuberculosis* belonging to different strain families induce different levels of cytotoxicity in human alveolar epithelial cells (A549) *in vitro*. We also investigated whether there was a difference in the cytotoxicity levels induced by the isolates grown in the presence of oxygen and absence of oxygen. Finally, we also addressed the question as to whether the level of toxicity was related to the formerly observed differences in adhesion and invasion of A549 cells.

METHODS

Strains

Fifteen clinical isolates of *M. tuberculosis* with different restriction fragment length polymorphism (RFLP) signatures were obtained from the culture collection of the TB section of the Department of Medical Microbiology and Infection Prevention and Control, University of KwaZulu-Natal, Durban. Three of the isolates belonged to the Beijing family, six to the F15/LAM4/KZN family (including three XDR isolates) and six were strains with unique fingerprint patterns. *M. tuberculosis* H37Rv (ATCC 27294) and H37Ra (ATCC 25177) were included as virulent and avirulent controls respectively.

Growth Conditions

Growth in the Presence of Oxygen

The isolates were inoculated in triplicate in Middlebrook 7H9 broth (Difco) and incubated with gentle agitation at 37°C in a shaking incubator. The cultures were used when an optical density (measured at 600nm) of 1 was reached, which took incubation periods between two to three weeks.

Growth in the Absence of Oxygen

The isolates were inoculated in triplicate in Middlebrook 7H9 broth (Difco) in screw-capped 15 ml tubes with a diameter of 17 mm containing 9 ml of broth. To obtain an oxygen deprived environment the cultures were incubated at 37°C in an upright position

without agitation for a period of three months [8, 12]. Thereafter, the bacterial cultures were adjusted to an OD (measured at 600nm) of 1 to equate the number of bacilli to those grown in the presence of oxygen.

Inocula Preparation

The broths were centrifuged (3000 x g, 10 min) and the supernatant discarded. The bacteria containing pellets were resuspended in 5 ml Eagle's Minimum Essential Medium (EMEM) (Biowhittaker-Lonza), vortex agitated for 5 min, and sonicated (10 w for 10 sec) to reduce clumping without killing the bacilli. To determine the number of colony forming units (CFU) per ml, 20 µl of four 10-fold serial dilutions of the suspension were plated out in triplicate on Middlebrook 7H11 complete agar media (Difco) containing 10% oleic acid, albumin, dextrose, catalase (OADC) and 0.05% Tween 80 (Sigma).

Epithelial Cell Line

The human type II alveolar epithelial cell line A549 (ATCC CCL 185) was used for the infection assay. The cells were maintained as previously described [8]. The monolayers were detached from the flask by trypsinization using trypsin-versene (Cambrex Bio Science). The trypan blue exclusion test was used to determine the number of viable cells. These were counted using a haemocytometer. One ml of suspension containing 10^5 cells was seeded into each well of a 24-well tissue culture plate. These were incubated for $\pm$40 h at 37°C in a 5% CO_2 atmosphere before being used for the experiment.

Infection Assay

The cells were washed thrice with phosphate buffered saline maintained at room temperature (PBS pH 7.3; Oxoid), after which EMEM (maintained at room temperature) was added into each well. Following inoculation of the cells with *M. tuberculosis* suspension at an m.o.i. (multiplicity of infection) of 9–12 bacteria per epithelial cell, the cells were incubated at 37°C in a 5% CO_2 atmosphere for 4 h. Control wells were included following the manufacturers instruction.

Measurement of Lactate Dehydrogenase release

The release of the cytosolic enzyme lactate dehydrogenase (LDH) from the infected A549 cells was measured using the CytoTox96 non-radioactive cytotoxicity assay (Promega) – a colorimetric kit and the manufacturer's instructions were followed with a

slight modification. Briefly, 50µl supernatant from each well (***M. tuberculosis* inoculum** represented the effector spontaneous; **uninfected A549 cell** represented the target spontaneous; **uninfected lysed A549 cells** represented the target maximum and **infected A549 cells** represented the experimental) was transferred into designated wells of a 96 well tissue culture plate and the CytoTox 96 kit components were added. LDH activity resulted in the conversion of a tetrazolium salt into a red formazan product. The intensity of the colour formed was directly proportional to the number of cells lysed. The absorbance of each designated well was measured at A_{450} (Glox Max). The average absorbance value of the culture medium (EMEM) background was subtracted from each average value of the different readings to normalize them. The percentage of cytotoxicity were then calculated using the formula in the assay protocol stated below:

$$\% \text{ Cytotoxicity} = \frac{\text{Experimental} - \text{Effector Spontaneous} - \text{Target Spontaneous}}{\text{Target Maximum} - \text{Target Spontaneous}} \times 100$$

Statistical Analysis

All data were analysed initially by one-way analysis of variance (ANOVA) and thereafter Dunn's multiple comparisons test with statistical significance (*P*) determined. Spearman's correlation coefficient (r_2) was calculated to determine the correlation between pairs of previous and present studied virulence mechanisms. A correlation was considered: perfect if r_2 is 1, strong as r_2 approaches 1, intermediate if r_2 was close to 0.5 and weak if r_2 was close to zero. All experiments were performed in triplicate and repeated three times.

Ethic Approval

This study was approved by the Biomedical Research Ethics Committee of the University of KwaZulu-Natal (Reference no: H039/06).

RESULTS

All the 15 clinical and the 2 laboratory isolates induced cytotoxicity in the A549 cells, but at different levels (Figure **1**). The growth conditions under which the isolates were cultured before the infection assay influenced the level of cytotoxicity they induced in the A549 cells. The isolates grown in the absence of oxygen induced a higher percent of cytotoxicity, when compared with the same isolate that was grown in the presence of oxygen (*P*<0.0001).
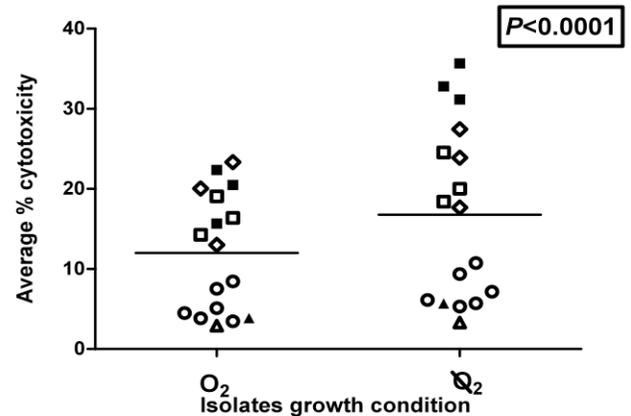
Figure 1: Comparison of induced cytotoxicity in A549 cells by *M. tuberculosis* isolates grown in O_2 (oxygen) and $\varnothing_2$ (no oxygen). Each point represents the average of the results obtained from three experiments performed in triplicate for each *M. tuberculosis* isolate.

◇ Beijing isolates.

■ F15/LAM4/KZN (XDR variant) isolates.

□ F15/LAM4/KZN isolates.

○ Isolates with unique DNA fingerprint.

▲ H37Rv.

△ H37Ra.

P<0.0001.

Isolates Cultured in the Presence of Oxygen

The cytotoxicity induced by organisms grown in the presence of oxygen ranged from 14.3% to 22.4% for the F15/LAM4/KZN strains, and 13% to 23.3% for the Beijing strains (Table **1**). The isolates with unique DNA fingerprint induced between 3.5% and 8.5% toxicity, while for the laboratory strains the values were 3.9% (H37Rv) and 2.9% (H37Ra) (Table **1**). Two of the Beijing and four of the F15/LAM4/KZN family members induced levels of cytotoxicity significantly higher than that of the virulent laboratory strain (H37Rv) (*P*<0.05). Two of the three Beijing and all the six F15/LAM4/KZN isolates also induced levels of cytotoxicity that were significantly higher than that of the avirulent laboratory strain (H37Ra) (*P*<0.05), which induced the lowest level of cytotoxicity (Table **1**). Each experiment was performed three times in triplicate and the coefficient of variation (CV) was within acceptable range (Table **S1**).

Isolates Cultured in the Absence of Oxygen

The Beijing and F15/LAM4/KZN isolates that were grown in the absence of oxygen induced cytotoxicity in A549 cells ranging from 17.7% to 27.5% and from

Table 1: **Induced Cytotoxicity in A549 alveolar epithelial cells by *M. tuberculosis* isolates grown in the presence and absence of oxygen**

M. tuberculosis Strains	Average % Cytotoxicity				
	Oxygen Present		Oxygen Absent		
	(mean ± SD)	$P^{\S}$	(mean ± SD)	$P^{\S}$	
Beijing 1	20.1±0.76	***††	23.9±0.86	*†††	
2	23.3±0.13	***†††	27.5±0.31	***†††	
3	13±1.63		17.7±1.01		
XDR-KZN 1	15.7±0.57	††	31.2±0.74	***†††	
2	20.5±0.22	***†††	32.8±1.89	***†††	
3	22.4±0.29	***†††	35.7±0.95	***†††	
KZN 1	14.3±0.53	†	18.4±0.43	†	
2	16.4±0.10	*†††	20±0.65	††	
3	19.1±0.20	**†††	24.5±0.68	*†††	
UNIQUE 1	5.1±0.34		7.2±0.63		
2	3.5±0.28		5.3±0.14		
3	8.5±0.71		10.7±0.37		
4	4.5±0.41		6.1±0.47		
5	3.8±0.26		5.7±0.25		
6	7.5±0.39		9.4±0.23		
H37Rv	3.9±0.34		5.7±0.26		
H37Ra	2.9±0.46		3.3±0.03		

Epithelial cells were exposed to the various bacterial inocula for 4h at 37°C. The results are the mean of three experiments performed in triplicate. SD, standard deviation; $P<0.05$(*), $P<0.01$(**), $P<0.001$(***) as compared with H37Rv; $P<0.05$(†), $P<0.01$(††), $P<0.001$(†††) as compared with H37Ra.

18.4% to 35.7% respectively (Table **1**). The percent cytotoxicity induced by isolates with unique DNA fingerprint ranged from 5.3% to 10.7% while that of the laboratory strain H37Rv was 5.7% and that of the H37Ra was 3.3% (Table **1**). The highest cytotoxicity level (35.7%) was induced by an XDR isolate of the F15/LAM4/KZN strain family (Table **1**). The mean cytotoxicity induced by the three Beijing and the XDR-F15/LAM4/KZN isolates were higher than the H37Rv ($P<0.05$ and $P<0.01$ respectively). There was a significant difference in the percent cytotoxicity induced by the XDR-F15/LAM4/KZN isolates when compared to the isolates with unique DNA fingerprint ($P<0.05$). The avirulent strain (H37Ra) was observed to have induced the lowest level of cytotoxicity among the isolates and the difference was significant when compared to two of the Beijing ($P<0.05$) and all of the F15/LAM4/KZN ($P<0.05$) family members. Each experiment was done three times in triplicate and the coefficient of variation (CV) was within acceptable range (Table **S2**).

Comparison between Isolates Grown with and without Oxygen Present

Isolates that had been grown in the presence of oxygen induced a lower level of cytotoxicity after exposure to A549 cells when compared to when the isolates were grown in the absence of oxygen (Figure **1**) ($P<0.0001$). We observed that the induced level of cytotoxicity of isolates with unique DNA fingerprints clustered with the two laboratory controls (H37Rv and H37Ra) under both growth environments (Figure **1**).

Relationship between Adherence, Invasion and Cytotoxicity of the Isolates

To determine whether a relation existed between adherence and invasion and toxicity we compared the toxicity results obtained from this study with the adherence and invasion results from our previous studies [7, 8] using the Spearman's correlation coefficient (r_2). A strong and significant positive correlation was observed between cytotoxicity and adherence ($r_2 = 0.939$ – oxygen present) as well as cytotoxicity and invasion ($r_2 = 0.956$ – oxygen present; $r_2 = 0.939$ – oxygen absent) (Table **2**).

DISCUSSION

The hallmark of pulmonary tuberculosis is damage to alveolar tissue. Several research groups have studied the toxic effect of *M. tuberculosis* on alveolar

Table 2:   Spearman's Correlation Coefficient (r_2) Values between Different Pairs of Virulent Mechanisms under Different Isolate Growth-Environment

Growth Environment	Virulent Mechanism	
	Cytotoxicity vs adherence	Cytotoxicity vs invasion
Oxygen present	0.939	0.956
Oxygen absent	ND	0.939

ND (not done).

macrophages and alveolar epithelial cells [5, 10, 13, 14]. We previously reported on the ability of strains belonging to the Beijing and F15/LAM4/KZN families of *M. tuberculosis* grown in the presence of oxygen and absence of oxygen to adhere to and invade A549 alveolar cells [7, 8]. Here, we report on different induced levels of cytotoxicity in alveolar epithelial cells by clinical isolates of *M. tuberculosis* that, based on their RFLP signature, belong to different strain families.

The high level of induced cytotoxicity that we observed in the A549 cells by the isolates belonging to the Beijing and F15/LAM4/KZN in comparison to H37Rv and isolates with unique fingerprints, confirms the high virulence capacity of these successful families of strains. This is in keeping with earlier reports on differences in virulence of *M. tuberculosis* strains [15-17]. This also agrees with the report by McDonough and Kress [5], that cytotoxicity is specific to virulent mycobacteria.

Our observation that the XDR isolates of the F15/LAM4/KZN family induced the highest level of cytotoxicity suggests that XDR variants of this family may cause increased tissue destruction compared to the other isolates. This attest to its virulence and may have contributed to the high mortality rates of patients infected with this strain in KwaZulu-Natal province of South Africa as reported by Gandhi *et al.* [18].

A number of studies have been done on the effect of *M. tuberculosis* on alveolar macrophages [14, 19]. These studies reported that infection of macrophages with *M. tuberculosis* triggered apoptosis [14, 19, 20], which is a programmed cell death. This might result in the killing of the bacilli present in the apoptotic cell, when these cells are ingested by other macrophages [20]. It was further suggested that this is accompanied by antiapoptotic mechanisms [21]. Most of these studies used one virulent laboratory strain of *M. tuberculosis* (H37Rv). Danelishvili *et al.* [14] reported that H37Ra induced a higher level of apoptosis in macrophage cells than the H37Rv strain.

Reports have also been published on the effect of *M. tuberculosis* on alveolar epithelial cells following infection with one of the virulent laboratory strains. These all suggested that in contrast to apoptosis that had been observed in alveolar macrophages, necrosis was observed in alveolar epithelial cells [10, 14], which is an accidental cell death. The infection of alveolar epithelial cell with *M. tuberculosis* was also reported to be associated with apoptosis inhibition [14].

That the isolates grown in the absence of oxygen had a more virulent phenotype by being more invasive and inducing higher levels of necrotic cytotoxicity in comparison to the same isolates grown in the presence of oxygen is in line with our previous findings of increased invasive abilities of isolates grown in the absence of oxygen [8].

In this and our previous studies the Wayne approach [12] was used to achieve an oxygen-depleted environment, as currently there is no gold standard to achieve this condition *in vitro*. This approach allows for a self-generated depletion of oxygen, which has been reported to result in drastic changes in the bacilli energetic and metabolic status [22, 23]. In which the transcriptional expression of *M. tuberculosis* bacilli grown under oxygen-depletion have similarities with the transcriptional expression of bacilli response to mouse lung immunity [22]. This may further explain our previous report on the increased invasiveness of bacilli grown under oxygen-depleted environment [8].

We show a significant positive correlation between cytotoxicity and adherence, as well as cytotoxicity and invasion. From this, we conclude that invasion is a post adherence event, that leads to cytotoxicity and that the adherence of the bacteria to the host cell is a prerequisite for invasion to occur as reported for other bacteria [24, 25].

In conclusion, we show for the first time that Beijing and F15/LAM4/KZN isolates of *M. tuberculosis* are cytotoxic to alveolar epithelial cells *in vitro* and that this cytotoxicity is upregulated in more invasive organisms

grown in the absence of oxygen. Further studies are required to determine the virulence of these isolates at a molecular level.

ABBREVIATIONS

XDR, LDH, TB, MDR, HIV, A549, RFLP, EMEM, OADC, ANOVA, CV, ND.

COMPETING INTEREST

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

OTA contributed to project conception and design, carried out experiments, involved in analysis and interpretation of data, drafting manuscript and revising it critically for important intellectual content. **AWS** contributed to project conception and design, involved in analysis and interpretation of data, drafting manuscript and revising it critically for important intellectual content. All authors read and approved the final manuscript.

SUPPLEMENTAL MATERIALS

The supplemental materials can be downloaded from the journal website along with the article.

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