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Phages specific for mycobacterial lipoarabinomannan help serodiagnosis of tuberculosis

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SUMMARY

This study evaluated the possibility to use six phages specific to the *Mycobacterium tuberculosis* lipoarabinomannan (LAM) as tools for tubercular serodiagnosis. We analysed sera samples from 30 subjects with active tuberculosis (TB+), 30 with latent tubercular infection (LTBI) and 60 healthy subjects as controls (K). Our data indicated a good antibody response of the TB+ and LTBI patients against the phage $Ri(7)_{17}$; the optical density (OD) values obtained from sera patients was statistically significant when compared to the control samples. Our results confirm that phage display technology might be useful to develop new tools for diagnosis of tuberculosis.

KEY WORDS: Mycobacterium tuberculosis, Tubercular serodiagnosis, LTBI (latent TB infection)

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Tuberculosis (TB) remains one of the major health problems in the world. Today we are missing a full understanding of the biology of *Mycobacterium tuberculosis*, the etiological agent of the tubercular disease, which has the ability to persist for long periods inside the host. However, it has been demonstrated that the *M. tuberculosis* cell envelope is a key factor in tuberculosis pathogenicity (Gevorkian *et al.,* 2005; Riley, 2006; Schluger *et al.,* 1998).

The immune system of the host infected by *M. tuberculosis* reacts with the activation of both T and B cells (Chiang *et al.,* 1997; Raja *et al.,* 2002; Scarpellini *et al.,* 2006). Different antigens that activate this type of immune response were discovered and many of these are proteins (Ewer *et al.,* 2006; Parra *et al.,* 2006; Rosseels *et al.,* 2006; Skjot *et al.,* 2000) that could be utilised as diagnostic tools (Scarpellini *et al.,* 2006). However the immune response against TB is also induced from other non-protein molecules, such as the cord factor, glycolipides and lipoarabinomannan (LAM). LAM is an important glycolipid of the mycobacterial cell envelope, that has immunological activity and that has been used as a diagnostic agent (Cunto-Amesty *et al.,* 2003; Sharma *et al.,* 2006; Tessema *et al.,* 2002).

Identification of epitopes for this molecule and their role in the pathogenesis of TB is difficult to define because the methods needed for their purification are very complicated. Functional characterization of genes involved in their synthesis is almost impossible. With the development of phage display technology, it has been possible to produce phages that mimic epitopes of both proteins and non-protein antigens, that could be used in the diagnosis of TB and other diseases in place of different antigens (Petty *et al.,* 2007; Sharma *et al.,* 2006).

The aim of our study was to confirm whether phages mimicking epitopes of the LAM previously identified (Sharma *et al.,* 2006) might be used for serodiagnosis of TB.

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This study enrolled 3 different groups of patients: TB Positive group (TB+) comprising 30 subjects with active TB; Latent TB Infection group (LTBI) of 30 subjects with latent tubercular infection and a control group (K) comprising 60 healthy subjects. In diseased subjects, TB was demonstrated with the isolation and identification of *M. tuberculosis* from the biological samples of these patients. For the LTBI group the latent infection was demonstrated by a positive reaction to purified protein derivative (PPD) and by the Quantiferon TB gold (QFT-G) assays. The latter was used because it can specifically recognise subjects infected by *M. tuberculosis* . None of subjects of the LTBI group developed active TB. In the control group both the indirect tests were performed and all subjects were negative to QFT-G but 8 of them were PPD positive (possibly as a consequence of preceding BCG vaccination).

Blood samples were taken from all subjects and sera were separated for the ELISA assays and to determinate the presence of IgG antibodies against the phages.

Different phages previously reported (Sharma *et al.,* 2006) were tested namely, $Ri(7)_{19}$ (Met-Pro-Phe-Val-Thr-His-Asn) $Ri(7)_1$ (Met-Ser-Pro-Arg-Ala-Thr-Ile) $Ri(7)_{24}$ (Tyr-Met-Asp-Leu-Gly-Met-Lys) $Ri(7)_{17}$ (Ser-Met-Met-Thr-Glu-Leu-Leu), $Ri(7)_{22}$(Asn-Leu-Thr-Asp-Ile-Leu-pro) and Cs35. Their purification was carried out as described previously (Sharma *et al.,* 2006).

Antibody levels were determined with an enzyme-linked immunosorbent assay (ELISA) (Zanetti *et al.*, 2005). Briefly, the microtiter plates were coated with 0.05 ml of phage diluted in calcium carbonate buffer (pH 9.6) of the strength 0.1 M to obtain a phage concentration of 10^9 PFU/well After an overnight incubation at 4°C, the plates were blocked for 30 min in a 5% solution of skim milk in phosphate-buffered saline (PBS, pH 7.2). Human sera diluted 1:20 in PBS/Tween (PBS and Tween 20 at a concentration of 0.05%) were placed in the wells, and the plates were incubated at room temperature for 2 h. The plates were washed five times with PBS/Tween, and 0.1 ml of anti-human IgG (Sigma-Aldrich) diluted 1:1000 in PBS/Tween was added to each well. After 1 h incubation, the plates were washed five times with PBS/Tween, and 0.1 ml of p-nitrophenylphosphate substrate (Sigma-Aldrich) was placed in each well. When sufficient color had developed, optical density was measured at 405 nm (OD_{405nm}) with the VERSA Tunable Max microplate (Molecular Device, Ismaning/Munich, Germany).

The arbitrary cut-off was chosen considering the median value of the OD_{405nm} observed in the TB and LTBI group. The statistical analyses were performed by the chi square tests with Yates corrections. We observed no reactivity pertaining to the phages $Ri(7)_{19}$, $Ri(7)_1$, $Ri(7)_{24}$ and Cs35 in the three groups that we analysed (Fig. 1 and Tab. 1). However, against the $Ri(7)_{17}$ phage (Fig. 1 and Tab. 1) the TB+ group showed a good humoral response with OD > or equal to 0.5 nm in 17 sera out of the 30 (57%) analyzed; in the LTBI Group a positive response was recorded for 20 out of 30 (67%) sera and in the control group only 12 samples out of 60 (20%) reacted to this phage.

On the other hand, $Ri(7)_{22}$ phage (Fig. 1 and Tab. 1) positively reacted with 11 samples (37%) corresponding to subjects with active disease (TB+), 18 (60%) sera from subjects with LTBI and with 30 (50%) sera of the healthy subjects with an OD 405 nm > or equal to 0.5.

The Chi-square test performed indicated that the immunoreactivity against the $Ri(7)_{17}$ phage was observed at a statistically significant difference both in the group of subjects with active TB versus the control group (p=0.0011 and odd ratio=5.23) and in the LTBI group versus the control group (p=0 and odd ratio=8) (Table 1). No difference was observed between TB+ and LTBI groups (p=0.59 and odd ratio=0.65). Also, no significant difference was recorded among the humoral responses against the $Ri(7)_{22}$ phage for the active TB group and the control group (p=0.33 and odd ratio 0.58) and between the LTBI group and control group (p=0.5 and odd ratio=1.50) (Table 1) and between the TB+ and LTBI (p=0.12 and odd ratio=0.39) groups.

No difference was observed in the responses registered against the $Ri(7)_{17}$ phage versus $Ri(7)_{22}$ in the active TB group (p=0.19 and odd ratio=0.44) as well as LTBI group (p=0.78 and odd ratio=0.75).

Our study investigated two phages corresponding to the epitopes of LAM, as described previously (Sharma *et al.*, 2006), to assess if they could be used to diagnose TB. We observed statistically significant differences against the phage $Ri(7)_{17}$ between the reactivity in the group of subjects

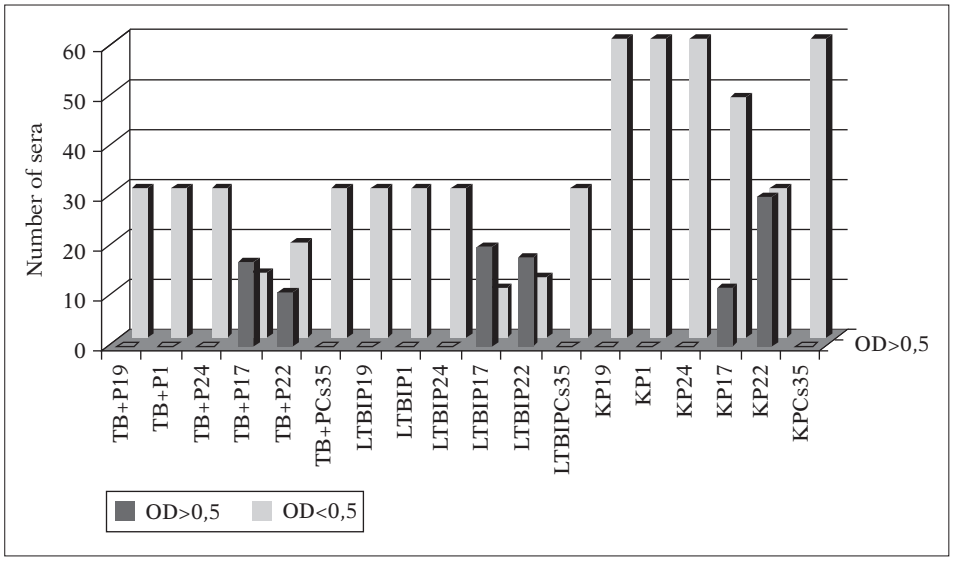

FIGURE 1 - *Humoral response against the phages Ri(7)19, Ri(7)1, Ri(7)24, Cs35, Ri(7)17 and Ri(7)22 in the TB+, LTBI and K Groups.*

TABLE 1 - *Comparison of phages mimotopes performance for three groups of subjects analyzed in our study. In the table were indicated the p value for comparison between TB+ and LTBI Groups with K Group (Control Group).*

Phages	*TB+ (N*=30)*		*LTBI (N*=30)*		*K (N*=60)*	
	+	-	+	-	+	-
Ri(7)19	0	30	0	30	0	60
Ri(7)1	0	30	0	30	0	60
Ri(7)24	0	30	0	30	0	60
Cs35	0	30	0	30	0	60
Ri(7)17	17 (57%) p=0,0011	13 (43%)	20 (67%) p=0	10 (33%)	12 (20%)	48 (80%)
Ri(7)22	11 (37%) p=0,33	19 (63%)	18 (60%) p=0,5	12 (40%)	30 (50%)	30 (50%)

N*= number of samples

with active TB and LTBI versus the control group. Unfortunately, our data indicated that this phage was not able to discriminate between active and latent tubercular infection. Nonetheless, in the active TB group a good humoral response was observed in 17 samples (57%) and in the LTBI group this type of response was observed in 20 patients (67%). These results might appear to be skewed due to the low number of the samples analyzed. Therefore, we recommend that the phage be tested against a large number of subjects to discriminate between the two forms of tubercular disease.

Against the second phage $Ri(7)_{22}$, no statistically significant difference was observed in the reactivity of the control group compared with the humoral response observed in the TB and LTBI patient groups. In fact, thirty sera of the control group reacted against the $Ri(7)_{22}$ phage (50%), whereas against the $Ri(7)_{17}$ phage a positive humoral response was recorded in only 12 samples (20%). This may be due to the fact that BCG vac-

cination status often overshadows serodiagnostic efficacy of different antigens. This was indeed true for the 8 subjects in our control group who had been BCG vaccinated previously, and so the reactivity observed for both phages in these subjects might be due to the vaccination. Positivity recorded among the other control patients might be a consequence of cross-reactivity with environmental mycobacteria.

Finally, our data indicate that the phage $Ri(7)_{17}$ could be a potential tool in the serodiagnosis of tuberculosis and that the use of phages may be advantageous (being easy amplified and shipped) in comparison to other tools such as the development of monoclonal antibodies (Petty *et al.,* 2007). However, our observations as reported may not be construed as advocating field level application of the phage. We recommend it to be tested against a large number of samples and those originating from different infection backgrounds and different geographic locations. Our findings nonetheless serve as an important milestone in developing phage-based assays for tuberculosis.

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