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Tuberculosis skin testing, anergy and protein malnutrition in Peru

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SUMMARY

SETTING—Malnutrition and intestinal parasites cause immunosuppression. This may cause false-negative tuberculin skin tests (TST) and failure to identify tuberculosis (TB) infection.

OBJECTIVE—To assess factors associated with TST positivity and anergy in disadvantaged communities in Peru.

DESIGN—A study of 212 randomly selected adults: 102 in a rural Amazonian village and 110 shanty town residents in urban Lima.

RESULTS—Respectively 52% and 53% of urban and rural jungle populations were TST-positive. Using simultaneous tetanus and candida skin tests, 99% had at least one positive skin test. Generalised anergy was therefore rare, despite frequent intestinal parasitic infection, including 34% helminth infection prevalence in the jungle. TST positivity was associated with age ($P = 0.001$), known TB contact ($P = 0.02$) and poor household ventilation ($P = 0.007$). TST positivity was not significantly associated with crowding, reported past TB, single/multiple BCG vaccination, income, intestinal parasites, dietary factors, body mass index or body fat. Individuals with lower anthropometric body protein, as measured by corrected arm muscle area, were less likely to be TST-positive ($P = 0.02$), implying that protein malnutrition caused tuberculin-specific anergy.

CONCLUSION—These results identify the importance of household ventilation for community TB transmission and add to the evidence that protein malnutrition suppresses TB immunity, causing false-negative TST results.

Keywords

tuberculosis; anergy; protein malnutrition; anthropometry; TST

One third of the world population are estimated to be infected with tuberculosis (TB), a condition that annually causes 2 million deaths.[1] The tuberculin skin test (TST) or Mantoux test is most widely used to test for TB infection. It constitutes an intradermal injection of

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tuberculin (purified protein derivative) followed by measurement of the resultant induration 48–72 h later. A positive TST indicates active or latent infection with *Mycobacterium tuberculosis*, although infection with environmental mycobacteria can cause false-positive reactions.[2]

The TST relies on a competent delayed-type hypersensitivity reaction to the injected tuberculin. Generalised or antigen-specific immunosuppression (anergy) may cause false-negative TST.[3] Anergy can be caused by many factors,[2] including malnutrition[4,5] (particularly protein[6,7] and micronutrient[8] deficiency), intestinal parasitic infection (particularly helminths),[9,10] chronic disease[11,12] and human immunodeficiency virus (HIV).[3,4] As TB is most common in regions where these factors are also common, this could lead to considerable underdiagnosis of TB infection. Although HIV is of low prevalence (0.5%) in Peru,[13] poverty and malnutrition[14] are common. Furthermore, intestinal parasitic infection is endemic.[15,16] Despite this, the majority of adults in Lima have previously been found to be TST-positive.[17,18]

TB infection may be underdiagnosed if malnutrition and parasitic infections cause false-negative TST results. We therefore studied the potential determinants of TST positivity and anergy in two disadvantaged Peruvian populations, chosen because of nutritional, socio-economic and parasitic heterogeneity.

STUDY POPULATION AND METHODS

Subjects and ethics

Study families were randomly selected from recently collected census data from two Peruvian populations. Fifty-three households were visited in Flores de Villa, a shantytown in the desert on the outskirts of southern Lima, and 39 families were visited in Santo Tomas, a village 5 km from the Amazonian city of Iquitos. Adults over 15 years of age who gave informed written consent joined the study. Exclusion criteria were pregnancy ($n = 3$) and current TB treatment ($n = 0$), as these would confound some results. In total, 33% of eligible adults did not take part due to disinterest or unavailability. This resulted in sample groups of 110 individuals from the city and 102 from the jungle.

Approval was obtained from the ethical review board of Asociación Benéfica PRISMA, Lima, Peru. All those with symptomatic parasitic infections received treatment at the local health centre free of charge.

Questionnaires

Investigators completed questionnaires concerning TB risk factors, measures of families' socio-economic status, vaccination history and diet in the last week, including alcohol consumption. Household ventilation was assessed as poor if construction techniques prevented the free flow of air through the building. Known contact of any duration with a TB patient was recorded, and whether this had occurred in the home, family or workplace.

Skin tests

To test for anergy, each participant had three simultaneous intradermal injections: tuberculin (0.1 ml, 5TU,* Avensis Pasteur, Toronto Canada; superior radial aspect of the right arm), tetanus toxoid (0.1 ml, 2 Lf units, ‘Anatoxal Te’, Berna Biotech, Bern, Switzerland; proximal radial surface of the left arm) and *Candida albicans* antigen (0.1 ml; distal radial

*5TU is the standard dose used in most of the Americas, including Peru, where this research took place, and the United States. 5TU was also the dose used in our previous research, upon which these experiments were based and with which we compared our results.

surface of the left arm). Two makes of candida antigen were used (Hollister-Stier, Spokane, WA, and 'Candin', Allermed, San Diego, CA, USA). The syringes (1 ml tuberculin syringes with 25G short-bevel needle, Terumo, Somerset, NJ, USA) were colour coded so that the field workers were blinded to the contents.

The results of the intradermal injections were read by trained field workers using the ball-point pen method[2,19] 48–72 h after placement. Most results were also read by one of the authors (TFP), and any disagreement resolved by repeating the measurement. TST reactions were considered positive if induration was >10 mm in diameter, as recommended for TB endemic populations such as these.[2] For candida and tetanus, a 5 mm induration cut-off was used.[3,17] The number of visible bacille Calmette-Guerin (BCG) scars was recorded. Smallpox scars were infrequent and were not recorded.

Anthropometry

Height and weight were measured using a wooden height scale and weight scales (Soehnle, Murrhardt, Germany). Anthropometric measurements were taken of the left acromio-olecranon length and the mid upper-arm circumference (MAC). The triceps skin-fold thickness (TSF) was measured in triplicate with skin-fold callipers (Holtain, Dyfed Ltd, Crynych, UK). The same investigator (TFP) took all the readings, which are operator dependent.[20,21] Body mass index (BMI) was calculated as kg/m^2. As an indicator of body protein content that is sensitive to chronic malnutrition,[20,22,23] the corrected (bone-free) arm muscle area (CAMA) was calculated as follows:[24]

$$\text{CAMA (males)} = \frac{\text{MAC} - \pi \times \text{TSF}^2}{4\pi} - 10$$

$$\text{CAMA (females)} = \frac{\text{MAC} - \pi \times \text{TSF}^2}{4\pi} - 6.5$$

The arm fat area (AFA) was used as a measure of total body fat. This is sensitive to acute malnutrition,[20] and was calculated as follows:

$$\text{AFA} = \frac{\text{MAC} \times \text{TSF}}{2} - \frac{\pi \times \text{TSF}^2}{4}$$

Stool samples

Single stool samples were requested from all participants and were stored in formaldehyde and concentrated by the method of Richie.[25] Microscopy was used to examine for the presence of intestinal parasites.

Statistical analysis

Data analysis was performed with a 95% confidence level, using the statistical software SPSS 11.5 (SPSS Inc, Chicago, IL, USA) and STATA 8.0 (Stata Corp, College Station, TX, USA). Analyses were corrected for multiple outliers using the test of Hadi. Fisher's exact test was used to test for differences in dichotomous variables between groups. The Mann-Whitney-U test was used to test for differences in continuous variables because these were not normally distributed. Univariate analysis was not considered robust because there were significant correlations between many of the potential cofactors of TST positivity. The non-

Gaussian distribution of TST induration prevented multiple linear regression. Potential predictors of TST positivity were therefore only analysed in a multiple logistic regression that modelled the probability of being TST-positive. Initially, all variables were included in a nested model. The least statistically significant variables were then removed one by one, using the likelihood ratio test, until only variables with statistical significance remained. The model included the universal confounders age, locale (i.e., city or jungle) and sex at all stages. Multiple linear regression was used to analyse factors associated with tetanus and candida skin test induration.

RESULTS

TB skin tests and associations

Fifty-two per cent of city inhabitants and 53% from the jungle had positive TST reactions (Figure 1A). The multiple logistic regression analysis of the assessed potential determinants of TST positivity is shown in the Table.

Age

As age increased, the TST was more likely to be positive (Figure 2A). With each decade >16 years of age, there was a 43% increase in the odds of being TST-positive.

TB contact

TST positivity was more likely in those who reported known past contact with a TB patient (Figure 2B). However, TB exposure increases with age. Controlling for this with multiple logistic regression indicated 2.4 times greater odds of TST positivity in those who reported past TB contact, independent of other factors (Table, $P = 0.02$).

Ventilation

No jungle dwellings were assessed to have poor ventilation, in contrast with 49% of those in the city, reflecting differences in climate and house building techniques. Multiple regression showed that those with a poorly ventilated house had 3.6 times greater odds of being TST-positive than those with adequate ventilation (Figure 2C, $P = 0.007$).

Other factors that could also have indirectly influenced TB contact were recorded, but none was statistically associated with TST positivity (Table). Specifically, there were no significant associations of locale, sex or income with TST positivity, despite mean earnings of <$1US/day in both populations.

Anthropometry

Those with lower CAMA, indicating lower body protein stores, were less likely to have a positive TST reaction. Figure 2D illustrates this relationship and shows 36% TST positivity in those from the lowest quartile of CAMA compared with 64% positivity in those from the top quartile. Multiple logistic regression demonstrated that this affect was independent of confounding factors (Table, $P = 0.02$), such that with every cm^2 rise in CAMA there was a 5% increase in the odds of being TST-positive. Neither BMI nor AFA were significantly associated with TST results.

Dietary factors

As can be seen in the Table, there was no link between TST positivity and frequency of consumption of meat, fruit or vegetables, dairy produce or the number of units of alcohol drunk each week.

Intestinal parasitic infection

Intestinal parasitism was common. Protozoal infection (principally *Entamoeba coli*, *Chilomastix mesnilii* and *Giardia lamblia*) was diagnosed in respectively 47% and 48% of the city and jungle population groups ($P = 0.9$) and helminth infection (principally whipworm and roundworm) in respectively 6% and 34% ($P < 0.001$). There was no significant association, however, between TST positivity and infection with either protozoa or helminths ($P = 0.4$ and $P = 0.8$). Furthermore, there was no association between TST positivity and infection with individual species of parasite, with only pathogenic species, or with the total number of species of protozoa or helminths infecting each person (data not shown).

BCG vaccination

Prior vaccination with BCG was almost universal: more than 90% of both populations had at least one BCG scar, and many individuals had multiple scars, up to six in total (both groups: median 2 scars per person, interquartile range 1–2, $P = 0.3$). It is noteworthy that despite an association between multiple BCG scars and TST positivity in univariate analysis, multiple logistic regression revealed that this association was not statistically significant: the apparent effects of BCG were explained by other factors in the model.

Candida and tetanus skin tests and associations

Candida induration (Figure 1B) showed a borderline increase with age ($P = 0.05$, odds ratio [OR] 0.06), and tetanus induration (Figure 1C) was significantly associated with female sex ($P < 0.001$, OR 2.2), younger age ($P = 0.04$, OR 0.2) and living in the city ($P < 0.001$, OR 2.2). There was no statistically significant difference between the two types of candida antigen ($P = 0.2$, Allermed mean induration 12.6 mm [SD 6.8, $n = 132$]; Hollister mean 11.3 mm [SD 6.1, $n = 73$]). Neither candida nor tetanus skin tests were associated with parasitic infection, any anthropometric marker, dietary factors or income in separate multiple linear regression analyses (data not shown).

Anergy

A Venn diagram of the TST, tetanus and candida positivity results is shown in Figure 1D. The majority of participants (>85%) had two or three positive reactions. Only two participants (1%) had negative reactions to all of the skin tests; our data did not identify reasons for this non-response.

DISCUSSION

Slightly over half of individuals from both populations were TST-positive, and <1% of both populations had negative skin test reactions to all three antigens. In this setting, therefore, generalised anergy to all three antigens was not a major cause of false-negative TST results despite frequent poverty and intestinal parasitic infection. This contrasts with a similar urban population studied in 1992, in which 10% of adults had generalised anergy to all three of the same antigens,[17] although that study did not include nutritional or anthropometric assessment.

We found increased rates of TST positivity in those who had known previous contact with TB patients, consistent with what is known about airborne transmission.[2] The association we demonstrated between TST positivity and increasing age is also well defined[17],[18] and is presumably explained by the cumulative probability of unrecognised TB exposure increasing with age. Greater transmission of TB in areas of poor ventilation through increased TB exposure has been shown to be important in hospital settings,[2],[26] but the role of household ventilation has been little studied. We found that TST positivity was

independently associated with poor household ventilation, probably through increased exposure to airborne bacteria. This is consistent with evidence of considerable household clustering of TB infection in recent shantytown studies,[27,28] and improves our understanding of the mechanisms of community transmission of TB.

Lower body protein reserves, as indicated by lower anthropometric muscle measurement (CAMA),[20,22,23] was associated with fewer positive TST results but was not linked with tetanus or candida skin test results. Relatively protein-deficient individuals did not, therefore, have generalised anergy because almost all had positive candida and/or tetanus antigen skin tests. Rather, the association between relative protein deficiency and negative TST implies antigen-specific anergy to tuberculin in those with lower body protein reserves (i.e., a selective suppression of tuberculin delayed-type hypersensitivity). Theoretically, reduced rates of latent TB in protein-deficient individuals are an alternative explanation, but this is unlikely because biologically there is no reason why low body protein should protect against TB exposure or infection. Antigen-specific anergy to tuberculin was shown in vitro[29] and in vivo[3,11,29] as long ago as 1967,[12] and is often found in studies of patients with chronic wasting diseases such as TB and the acquired immune-deficiency syndrome (AIDS).

Protein deficiency has been shown to be associated with impaired TB immunity through a variety of mechanisms in vitro and in vivo, both in animal models[7,8,30] and in humans.[31–33] For example, protein-deficient guinea pigs show tuberculin anergy and increased mycobacterial susceptibility compared with protein-nourished animals.[7] Similarly, in mice, increased TB susceptibility caused by protein malnutrition can be reversed by the reintroduction of a protein-rich diet.[30] In humans, low serum protein levels were associated with reduced TST induration after conditions of low protein intake[6] and nutritional support reversed this anergy.[5] Further, in a study of children (including anthropometric assessment of arm muscle area), protein, but not calorie deficiency, was associated with reduced TST positivity.[33] Protein nutrition therefore plays a role in maximising TB immunity and resistance.

There was evidence of significant malnutrition in these populations. Factors such as extreme poverty (average income <$1US/day) and poor food variety, with a diet based mainly on carbohydrates, suggested that malnutrition, including protein and micronutrient deficiency, was very likely in both populations. Anthropometric measures vary with race,[34–36] and this prevents direct comparison of anthropometric cut-off criteria between ethnically distinct groups. We therefore considered anthropometric data relative to those within our study participants.[21,34] Notwithstanding, according to the USA-derived NHANES[20] data set for CAMA measurements, respectively 12% and 13% of the city and jungle populations were below the fifth centile.

There is evidence that intestinal parasites, and particularly helminths, cause chronic immune system activation and associated immunosuppression.[9,37] Our data, however, did not support an association between parasites and TST results or anergy despite 34% of the jungle population having helminth infections.

BCG vaccination has been found to be almost universal in Peru, with reported rates of 83–87%.[17,18] We found that 60% of individuals had multiple BCG scars despite lack of evidence of benefit from multiple inoculation.[38] Our data did not show an association between TST positivity and single or multiple BCG vaccination, despite a theoretical possibility[2] and previously conflicting results.[39,40] The number of participants in our study, however, limited the statistical power to detect a significant effect of this factor. Some studies have found an association between BCG and TST in univariate analysis without

taking confounding factors, such as age and TB exposure, into account,[3,41] and these should therefore be interpreted with caution.

CONCLUSIONS

This study confirmed frequent TST positivity in disadvantaged rural and urban Peruvian populations, and demonstrated that generalised anergy to multiple skin test antigens was rare. In this setting of endemic TB, ventilation was associated with TST results, implying that poor household ventilation increased the risk of TB infection. Anthropometric evidence of lower body protein reserves was associated with reduced TST positivity. This finding adds to evidence that protein malnutrition suppresses the immune reaction to TB antigens. Furthermore, low body protein stores may cause false-negative TST results, and protein nutrition should consequently be considered when interpreting the TST.

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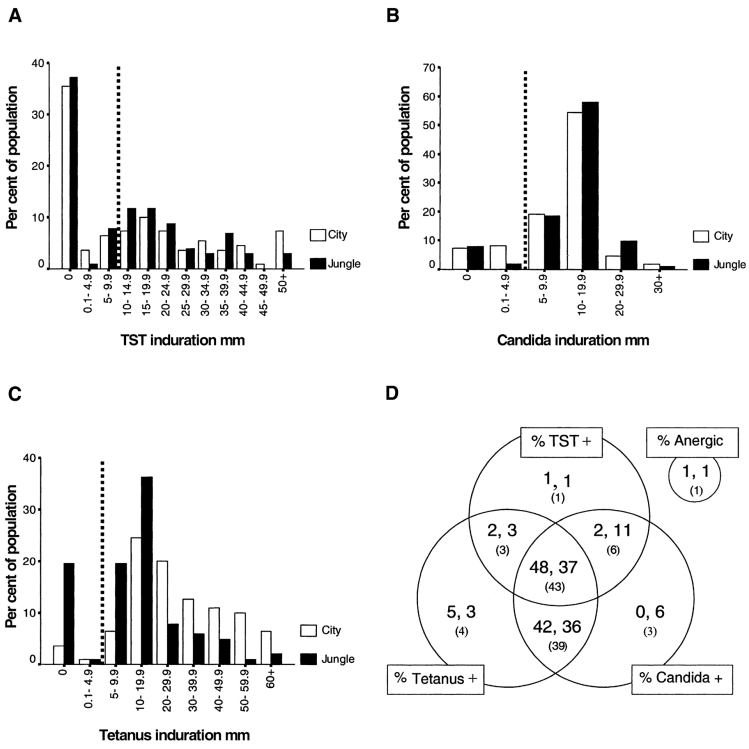

Figure 1.

Tuberculin, candida and tetanus skin test results for each population. **A.** TST results. **B.** Candida skin test results. **C.** Tetanus skin test results. The dotted line indicates the induration cut-off for test positivity in each case. **D.** Venn diagram of percentage positivity for each skin test in the city and jungle, respectively (combined population percentage in brackets). TST = tuberculin skin test.

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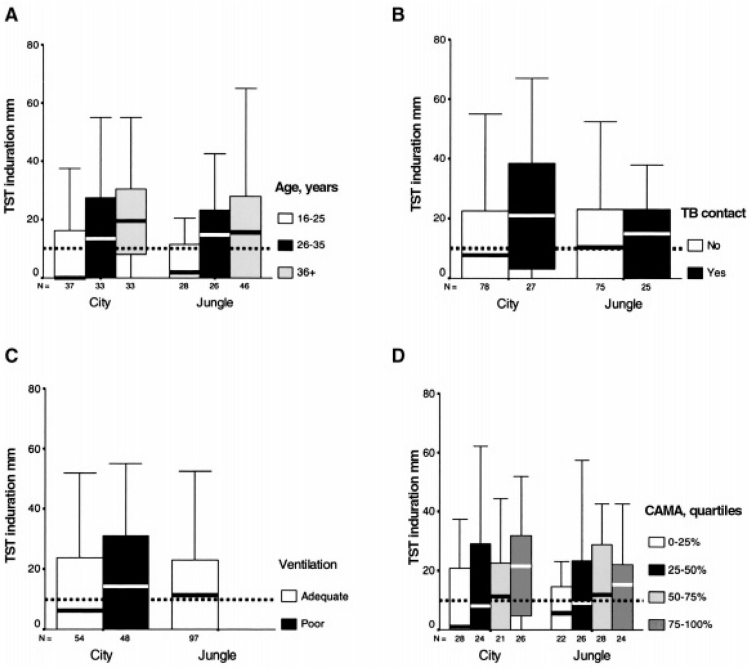

Figure 2.

Significant associations of TST positivity. TST results and **A.** age in decades; **B.** tuberculosis (TB) contact; **C.** household ventilation (no households in the jungle were assessed to be poorly ventilated); **D.** CAMA combined quartiles for each sex TST induration are shown in each graph with the inter-quartile ranges shown by boxes and the median by a central line. The dotted line indicates the 10 mm induration cut-off used for TST positivity. TST = tuberculin skin test; TB = tuberculosis; CAMA = corrected arm muscle area.

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Table

Potential associations of TST positivity in 212 subjects

Variable*	Measure	Subjects *n*	Multiple logistic regression		
			P value†	OR	95%CI
Factors associated with TB exposure					
Age	Years	210	0.001	1.04	1.02–1.07
Poor ventilation	Investigator assessed	49/206	0.007	3.56	1.41–9.00
Known TB contact	Participant reported	55/212	0.02	2.42	1.15–5.10
Past TB treatment	Participant reported	13/195	0.09		
BCG scars, *n*	None	18/212			
	1‡	68/212	0.5		
	2‡	79/212	0.4		
	≥3‡	47/212	0.1		
Household crowding	People per room	212	0.7		
Household death in last 2 years	Participant reported	20/210	0.8		
Nutritional factors					
CAMA	cm^2	204	0.02	1.05	1.01–1.10
BMI	kg/m^2	212	0.1		
AFA	cm^2	204	0.1		
Dairy consumption§	Days eaten/week	212	0.3		
Meat or fish consumption§	Days eaten/week	211	0.6		
Fruit/vegetable consumption§	Days eaten/week	211	0.6		
Alcohol consumption	Units/week	209	0.5		
Income	US$/day	192	0.7		
Parasitic factors					
Helminth infection	Stool microscopy	38/189	0.8		
Protozoal infection	Stool microscopy	90/189	0.4		
Universal confounders					
Locale	City (vs. jungle)	110/212	0.2		
Sex	Male (vs. female)	89/212	0.6		

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[*] Time since last BCG vaccination was not included in the model due to high colinearity with age.

[†] *P* values shown are those immediately prior to the variable being dropped from the model.

[‡] The BCG *P* values represent comparisons of 1, 2 or >3 scars with having no scar visible. No association with TST positivity was demonstrated when the number of BCG scars was considered as an ordinal variable (0, 1, 2 or ≥3 scars) or as a dichotomous variable (comparing 0 vs. ≥1; ≤1 vs. ≥2 or ≤vs. ≥3 scars) in any analysis.

[§] Diet was recorded as the number of days in the last week a food type was consumed. Carbohydrate was excluded due to >95% daily consumption. Dietary factors were analysed as the lowest quartile of consumers compared with the remaining 75%. ORs and 95%CI for age and CAMA are per year and per cm^2, respectively.

TST = tuberculin skin test; OR = odds ratio; CI = confidence interval; TB = tuberculosis; BCG = bacille Calmette-Guérin; CAMA = corrected arm muscle area; BMI = body mass index; AFA = arm fat area.