The frequency distribution of vitamin D Receptor fok I gene polymorphism among Ugandan pulmonary TB patients
[version 1; peer review: 1 approved, 2 approved with reservations]

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Abstract

Background: Mycobacterium tuberculosis (TB) is still a major problem globally and especially in Africa. Vitamin D deficiency has been linked to TB in the past and studies have found vitamin D deficiency to be common among Ugandan TB patients. The functional activity of vitamin D is dependent on the genotype of the vitamin D receptor (VDR) polymorphic genes. Recent findings have indicated that VDR polymorphisms may cause increased resistance or susceptibility to TB. The vitamin D ligand and its receptor play a pivotal role in innate immunity by eliciting antimicrobial activity, which is important in prevention of TB. The fok I vitamin D receptor gene has extensively been examined in TB patients but findings so far have been inconclusive.

Objectives: This study sought to investigate the frequency distribution of the VDR fok I gene polymorphisms in pulmonary TB patients and controls.

Methods: A pilot case control study of 41 newly diagnosed TB patients and 41 healthy workers was set up. Vitamin D receptor fok I gene was genotyped.

Results: The frequency distribution of fok I genotype in Ugandan TB patients was 87.8% homozygous-dominant (FF), 7.3% (Ff) heterozygous and 4.8% (ff) homozygous recessive. For normal healthy subjects the frequencies were (FF) 92.6%, (Ff) 2.4% and (ff) 4.8%. No
significant difference was observed in the FF and ff genotypes among TB patients and controls. The Ff heterozygous genotype distribution appeared more in TB patients than in controls. A significant difference was observed in the fok I genotype among gender p value 0.02. No significant difference was observed in ethnicity, p value 0.30.

**Conclusions:** The heterozygous Ff fok I genotype may be associated with TB in the Ugandan population.

**Keywords**
Vitamin D Receptor, Polymorphism, Fok I genotypes, Tuberculosis, Uganda

**Introduction**
Introduction
According to the World Health Organization (WHO) report on the ‘Use of high burden country lists for TB by WHO in the post-2015 era (WHO/HTM/TB/2015.29), Uganda was removed from the 22 high TB burden countries. However Uganda is still among the 41 high TB/human immunodeficiency virus (HIV) burden countries and TB remains a major public health problem. Although it is still unclear how individuals develop active TB there is emerging evidence of multi drug resistant TB (MDRTB). Genetic predisposition to TB has been suggested in several studies but the immunological associations with genetic polymorphisms are still unclear. Vitamin D insufficiency is common worldwide and recent studies have shown that vitamin D insufficiency is associated with a higher risk of active TB. The vitamin D receptor gene (VDR) has been identified as a candidate gene for TB susceptibility; however, studies reporting from different ethnic groups have been inconsistent. Various VDR polymorphisms have been identified and associated with TB susceptibility or resistance. Similarly the widely studied and functional single nucleotide polymorphism (SNP) rs2228570 of VDR fok I gene has shown variations. This polymorphism is due to the alteration in one of the start codons in which the amino acid thymine is replaced with cytosine (T/C). The dominant homozygous FF variant has high transcriptional activity with three amino acids less since translation starts at the second codon, while the homozygous recessive (ff) has 427 amino acids and is the longer form. The absence or presence of the restriction site is designated as F or f respectively. This study investigated the frequency distribution of VDR fok I gene among TB patients and compared it with controls in a Ugandan population.

Methods
After obtaining permission and informed consent (see ethical considerations) a pilot study in newly diagnosed smear positive TB patients and healthy controls was conducted between the months of April and June 2013 at the Mulago National Referral and Teaching Hospital located in North of Kampala, Uganda. By consecutive sampling adult patients who presented with persistent cough for more than 3 weeks had a positive Ziehl-Nielsen smear test for the first time and signed a written consent were considered eligible. Health workers and medical trainees who worked at the TB out patient’s clinic and other wards exposed to TB were matched for sex and age with the control group. All subjects were screened for human immunodeficiency virus (HIV) and data and consent forms were filled. Blood was then collected into tubes with ethylenediaminetetraacetic acid (EDTA) anticoagulant (Becton, Dickinson and company New Jersey USA) and whole blood samples were stored at 2–8°C before DNA column extraction.

Genotyping of fok I gene
Genotyping of VDR fok I gene was performed by PCR–direct sequencing method on ABI 310 sequencer. Human genomic DNA was extracted using the Genotype DNA isolation kit version 2.0 (HAIN Life Science, Germany) according to the manufacturer’s instructions. To check the purity genomic DNA was run on a 1.0% agarose gel in a 1 × Tris Acetate EDTA (TAE) buffer containing ethidium bromide for 1 hour at 120 volts and visualized under an UV transilluminator. The primer sequences used in our study were forward A 5’-GC TGG CCC TGG CAC TGA CTC TG TCT -3’ and reverse 5’-ATG GAA ACA CCT TGC TTC TCC TCC CTC as -3’ described by Harris et al. (1997). PCR was performed in 15.1 µl reaction volumes which contained 7 µl of PCR water, 1 µl of 25 mM MgCl₂, 1 µl of 10X master mix (Fisher biotec company Australia), 1 µl of 0.22 mg of each of the forward and reverse primers (Integrated DNA technologies company USA), 0.1 µl of 5U of Premixed Taq polymerase and finally 5 µl of pure DNA were added and the reaction was thoroughly mixed. The PCR GTQ Thermocycler programme involved an initial denaturation at 95°C for 5 minutes, followed by 30 cycles 70°C annealing for 45 seconds, elongation at 72°C for 1 minute and a final elongation at 72°C, for 10 minutes. Amplified DNA was purified using an extraction kit (JET quick company, USA) according to manufacturer’s instructions. Cycle sequencing PCR was performed using the Big Dye terminator KIT version 3, in the Thermocycler Gene Amp PCR system 9700. The cycling programme involved denaturation at 96°C for 1 minute for 1 cycle , annealing at 96°C for 10 minutes 30 cycles, elongation at 70°C for 30 seconds and a final elongation at 60°C for 5 minutes. The Dye EX 2.0 spin kit (250) (QIAGEN, Germany) was used to remove dye and other PCR products following the manufacturer’s instructions. Sequencing of the PCR products was done using the ABI Big Dye Termination kit (Applied Biosystems, USA) and the ABI prism 310 Genetic analyzer (Applied Biosystems). Sequences obtained were compared to those in the Gene Bank database by applying BLAST; NCBI tool. Finally DNA base sequencer assembly software version 4.7.0 bioinformatics tool was used for the Identification of fok I gene polymorphism. Polymorphism of FF genotype was identified on sequences with a start codon of ACG instead of an ATG followed by an ATG downstream. The homozygous ff had a start codon of ATG and consequently another ATG in the sequence. The heterozygous Ff genotype had an ACG, ACG and an ATG.

Statistical analysis
The sample size was calculated based on data of a previous study with mean of 78.3 for TB and 83.5 for healthy controls and a total of 82 samples at a power of 80% would detect an effect. We therefore divided the samples into cases and controls for a pilot study. Data were summarized into odds ratios, 95% confidence interval (95% CI), and alpha of p<0.05 was considered significant using STATA software (Stata Corp. STATA 12.0, College Station, Texas, USA). The frequency of vitamin D fok I gene was determined using percentages. A Chi square test was used to determine the allele and genotype frequency distribution and potential deviation from Hardy Weinberg equilibrium. Fok I genotypes were categorized as FF for dominant, Ff heterozygous and ff for homozygous variant.

Results
Description of social demographic characteristics of participants
A total of 82 participants were enrolled in the study. Of these, 41 had pulmonary TB while 41 were healthy subjects with no history of the disease. Majority of participants were males (66.0%). Among the participants with TB 73.2% were males while the females were 26.8%. The percentage of HIV/TB patients in this study of 24.4% was lower than the non HIV/TB patients. Thirteen (15.9%) of the 82 participants were HIV seropositive, of these three were from the control group. Most of the TB patients were drivers, farmers, teachers and others. Other lifestyle factors did not show significant
Variation. Details of the above results are shown in Table 1. Only four (10%) participants were aware of exposure to TB (data not shown).

Detection of fok I gene PCR amplicons and BLAST identification of fok I nucleotides

Following DNA extraction and amplification of the fok I gene in all 82 samples the electrophoresis results showed a band between 200 and 300 bps (Figure 1). The PCR products were then sequenced and a BLAST on the query sequences was done against those in the NCBI Gene bank to confirm the sequences of fok I. The gene product was named vitamin D3 receptor isoform VDRB1 vitamin D3 receptor isoform VDRA. It showed sequences between 238 bp and 260 bp with a percentage identity between 98–99% (see Dataset 1).

Frequency distribution of fok I genotypes and alleles among TB patients and controls

The frequencies of Fok I genotypes, FF, Ff, and ff in TB patients and in the controls were assessed. Table 2 shows the genotype and allele frequency distribution of this study population with their odds ratio, confidence intervals and p values. No significant difference was observed in the distribution of the homozygous genotypes in the study population and the two alleles. FF genotype was used as the reference and other genotypes were compared while the f allele was used as reference to the F allele (Table 2). The heterozygous Ff genotype was associated with TB, (OR 3.2) since it occurred more in the TB patients than the healthy subjects as shown in Table 2. There was a predominance in the allele distribution of F dominant verse the f recessive allele in both the TB and control group. Both HIV and non HIV individuals predominantly had the FF genotype.

Table 1. The social economic and demographic characteristics of 82 subjects.

<table>
<thead>
<tr>
<th>Socio-economic characteristics</th>
<th>TB patients, n, (%)</th>
<th>Healthy subjects, n, (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>30 (73.2%)</td>
<td>24 (58.5%)</td>
</tr>
<tr>
<td>F</td>
<td>11 (26.8%)</td>
<td>17 (41.5%)</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18 – 60 yrs</td>
<td>34.2 ±12.0</td>
<td>35.2 ± 10.7</td>
</tr>
<tr>
<td>Marital status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Single</td>
<td>18 (43.9%)</td>
<td>19 (46.3%)</td>
</tr>
<tr>
<td>Married</td>
<td>23 (56.1%)</td>
<td>22 (53.7%)</td>
</tr>
<tr>
<td>Shelter</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Large house</td>
<td>19 (46.3%)</td>
<td>25 (60.9%)</td>
</tr>
<tr>
<td>Small house</td>
<td>22 (54.6%)</td>
<td>16 (39.1%)</td>
</tr>
<tr>
<td>Employment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Employed</td>
<td>34 (83%)</td>
<td>28 (68%)</td>
</tr>
<tr>
<td>Unemployed</td>
<td>7 (7.1%)</td>
<td>13 (31.7%)</td>
</tr>
<tr>
<td>HIV status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>10 (24.4%)</td>
<td>3 (7.3%)</td>
</tr>
<tr>
<td>Negative</td>
<td>31 (75.6%)</td>
<td>38 (92.6%)</td>
</tr>
<tr>
<td>Daily alcohol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>5 (12%)</td>
<td>1 (2.5%)</td>
</tr>
<tr>
<td>No</td>
<td>36 (87.8%)</td>
<td>40 (97.5%)</td>
</tr>
<tr>
<td>Smoking</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>4 (10%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>No</td>
<td>37 (90%)</td>
<td>41 (100%)</td>
</tr>
</tbody>
</table>

Figure 1. Agarose gel showing PCR amplicons of Fok I gene in TB patients and controls. Lanes: M=100bp DNA Ladder, 1=Positive Control, 2=Negative Control, 3–7 and 8–13 = Representative sequencing PCR products from controls and cases respectively.
with only one HIV/TB patient having the recessive ff genotype. Hardy Weinberg equilibrium was tested to establish if the genotype and allele distribution among the TB patients and the control population was in equilibrium. The Chi square test estimated a deviation coefficient of 0.041 in the TB patients and 0.045 in the healthy subjects.

Genotype fok I distribution by gender

An analysis of fok I gene was performed to determine the frequency distribution of the fok I gene polymorphism among male and female subjects (Table 3). The frequency distribution of fok I FF homozygous dominant genotype was male was 57.3% for male and 32.9 % for females. There was one female with heterozygous Ff genotype while homozygous recessive ff genotype was only found male subjects. These results showed a significant difference in the distribution of the fok I genotypes among male and female subjects given a p value of 0.02. When a multivariate analysis was further performed against fok I, gender and other variables a p 0.01 was obtained.

Discussion

VDR polymorphisms have been studied in different populations and have shown various results. We document for the first time the frequency distribution of VDR polymorphism in the Ugandan population. This study reports a frequency of 87.8% of FF genotype in the Pulmonary Tuberculosis (PTB) patients; however previous studies have reported a 65% frequency of FF genotype among Indian PTB patients, 72% among the Venda of South Africa and 62% among west-Africans. The low frequency of f allele in this population is consistent with another study showing that the f allele of Fok I occurs less frequently in Africans compared to Caucasians and Asians, (Africans 24%, Caucasians 34%, and Asians 51%). The ff genotype frequency of 4.8% was similar to the one described for PTB patients in the African case control study of Gambia Guinea and Guinea Bissau. The ff genotype was reported to be 5% in an Indian healthy population, while it was 6% in the African American population and 3% in the Venda of South Africa. The ff genotype was only found among the males in this study. This observation differs from an Indian study showing the FF genotype only in males and a significant difference in the genotypic distribution between males and females. The VDR polymorphism was not in equilibrium with Hardy Weinberg and this could be attributed to the low frequency of the ff genotype. Among other studies that have also reported disequilibrium in the VDR are the studies in the three West African countries and a study on the Paraguay population. Disequilibrium observed in these

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**Table 2. Genotype and allele distribution of fok I gene among TB patients and controls.**

<table>
<thead>
<tr>
<th>Fok I Genotypes</th>
<th>TB patients, n = 41</th>
<th>HS, n = 41</th>
<th>OR 95% CI</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>FF</td>
<td>36 (87.8%)</td>
<td>38 (92.6%)</td>
<td>1.0 ref</td>
<td>0.81</td>
</tr>
<tr>
<td>Ff</td>
<td>3 (7.3%)</td>
<td>1 (2.4%)</td>
<td>3.2 (0.3-31.8)</td>
<td>0.33</td>
</tr>
<tr>
<td>ff</td>
<td>2 (4.8%)</td>
<td>2 (4.8%)</td>
<td>1.1 (0.1-7.8)</td>
<td>0.96</td>
</tr>
<tr>
<td>Allele</td>
<td>No of Allele in TB</td>
<td>No of Allele HS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>75 (91%)</td>
<td>77 (94%)</td>
<td>1.0 (0.9-1.1)</td>
<td>0.54</td>
</tr>
<tr>
<td>f</td>
<td>7 (8%)</td>
<td>5 (6%)</td>
<td>Ref</td>
<td>0.7</td>
</tr>
<tr>
<td>Total No of alleles</td>
<td>No in TB/Controls</td>
<td>No in TB/Controls</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>152 (92.7% )</td>
<td>12 (7.3%)</td>
<td>Ref</td>
<td></td>
</tr>
<tr>
<td>f</td>
<td>12 (7.3%)</td>
<td>12 (7.3%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

OR =Odds ratio, CI= confidence interval, FF reference genotype and f reference allele

**Table 3. Genotypes of fok I based on gender.**

<table>
<thead>
<tr>
<th>Fok I Genotypes</th>
<th>Male, n, (%)</th>
<th>Female, n, (%)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>FF</td>
<td>47 (57.3%)</td>
<td>27 (32.9%)</td>
<td>0.02</td>
</tr>
<tr>
<td>Ff</td>
<td>3 (3.7%)</td>
<td>1 (1.2%)</td>
<td></td>
</tr>
<tr>
<td>ff</td>
<td>4 (4.9%)</td>
<td>0 (0.0%)</td>
<td></td>
</tr>
</tbody>
</table>
studies may be due to genotyping errors but was overcome by use of high quality DNA which was achieved by secondary PCR. Other factors are genetic drift and mutations. There was no significant difference in the distribution of fok I genotype in different ethnic groups of our study population, (p value 0.30). However a difference was observed when the analysis was based on gender. The low numbers of females in this study may probably be explained by the female sex being a protection against TB according to a study. Among the 5 % to 10 % of individuals who develop active disease about 70% are males.

**Conclusion**

Although the frequency distribution of the homozygous fok I genotypes was not significantly different in the Ugandan TB patients and controls the Ff heterozygous genotype appears to be associated with TB in the Ugandan population. Therefore further studies are needed to elucidate this relationship.

**Data availability**


**Consent**

Written informed consent to participate in the study and publish these data was obtained from all participants.

**Ethical considerations**

Permission to carry out the study was obtained from the Research and Ethics Committee Mulago Hospital, (ref MREC: 329), the Institutional Review Board of the School of Biomedical Sciences Higher Degrees Research and Ethics committee, (ref SBS 108), and from the Uganda National Council of Science and Technology (ref No.1431). Before investigations, informed consent was obtained from the study subjects. Patients’ personal information was kept confidential by using serial codes instead of names on the questionnaire.

**Author contributions**

AE: conceived the study, AE and EJ: designed the experiments, AE, WW and EJ: developed proposal and was involved in research, MP contributed to study design and other statistics expertise AE WW, MP, KA and EJ prepared the first draft of manuscript. All authors were involved in the revision of the draft manuscript and have agreed to the final content.

**Competing interests**

No competing interests were disclosed.

**Grant information**

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The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Acknowledgement**

We would like to acknowledge the study participants, Mulago hospital and MBN laboratories for analysis.

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**References**


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✔ Range Nyagosya
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The vitamin D receptor plays an important role in the immune system and the roles have widely been studied. Several studies have been conducted to assess the frequency distribution as well as roles of Vitamin D’s in various diseases conditions; including; multiple sclerosis, cancer, diabetes and TB. However, the findings have been varying and inconclusive, imposing the need of carrying further investigations in different populations and settings to determine the roles and frequency distribution. It was on this varying state of Vitamin D that the authors, Ester et al, conducted a study in Uganda aiming to investigate the frequency distribution of the VDR gene (fok I) polymorphisms in pulmonary TB patients compared to controls in Ugandan population.

The article though short and with relatively small sample size of 82 (TB patients and health controls) is well written, appropriate description is given covering all sections; introduction, objectives methods, results, discussions and conclusion. The study found no significance difference in the frequency distribution of homozygous –dominant (FF) and homozygous receive (ff) genotype among TB patients and controls. However, heterozygous (Ff) genotype was more frequent among TB patients compared to controls (7.3% vs 2.4%). The author concluded that Ff gene may be associated with TB because it occurred more in TB patients than in health controls.

Specific comment:
In the discussion section the author gave references to other studies done elsewhere on the frequency of FF and ff genotypes, however, references on the frequency of heterozygous (Ff) is missing and should be added in the discussion section. In this study the difference was observed on this gene for the Ugandan studied population hence reference to other studies findings is important.

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of
expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 31 August 2016

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This manuscript describes a case-control study carried out at the Mulago National Referral and Teaching Hospital to assess the possible association between the fok I gene and TB diagnosis. The cases were defined as newly diagnosed cases of TB and were matched to TB negative controls taken from health workers at the hospital. The minor allele frequency (MAF) was smaller than expected with the overall MAF = 7%. While some differences were found between TB cases and controls, none were statistically significant. It is a fairly straight forward analysis and Dr. Acen and colleagues made a valiant effort to explore this question.

Some comments:

1. The controls were chosen from health workers and trainees at the hospital (age and sex matched). This brings in some confounding as health workers and medical trainees tend to be different than the general population in terms of education, SES, etc. some of which include risk factors for TB. This needs to be addressed in the discussion as a limitation. Also, were the controls tested to confirm they do not have TB, this should also be mentioned.

2. I am assuming the health workers are the controls, the wording is a bit strange. The manuscript needs to be proof-read by a native or more fluent English speaker

3. The authors state that cases and controls were matched on sex, but the numbers (table 1) do not show this. There are 30 males in the TB group and only 24 in the controls.

4. The main result of this paper is the OR of 3.2 presented in table 2. The authors claim that this is a real effect and shows a possible relationship between TB and the fok I gene. They neglect to mention the 95% CI that includes the null (0.3 – 31.8) in text which is misleading. With such small numbers and a non-significant effect this is not a meaningful finding. It argues that more research is needed, but it is hard to argue that there is a real effect shown in these data.

4. Statistical Analysis section and Table 2:

1. The authors list numbers (78.3 and 83.5) as means for use in a power calculation. What do these numbers stand for? If they are allele frequencies then that should be stated.
2. With such low frequencies in the some of the cells, a chi-square test is not appropriate. A Fisher's exact test should be used to determine p-values.

3. Why is FF used as a reference genotype but f used as reference allele? This makes the interpretation a bit more confusing.

4. How were the p-values calculated for Table 2? This needs to be indicated. What is the p-value for the reference groups from?

5. Table 3

1. The analyses based on sex needs to be described in the statistical analysis section.

2. What was the impetus to compare frequencies based on sex? It seemed to come out of nowhere.

6. The authors should focus more on the differences in MAF for fok I gene in their population compared to other published populations of different ancestry.

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.
In the Results it is claimed that “The heterozygous Ff genotype was associated with TB, (OR 3.2) since it occurred more in the TB patients than the healthy subjects. The Abstract also states that “The Ff heterozygous genotype distribution appeared more in TB patients than in controls”. And the discussion says “the Ff heterozygous genotype appears to be associated with TB in the Ugandan population”. I think these claims are misleading, as the result was not statistically significant at all. In fact, when you consider the raw numbers (3 of the 41 TB patients were Ff and 1 of the 41 healthy samples were Ff), I think it is clear that there is no evidence in this data for an association between Ff and TB status. I feel that the text should be revised to acknowledge the lack of statistical support for this claim.

2. Association between genotype and gender

The section "Genotype fok I distribution by gender" and Table 3 is not very clear. It appears to state that genotype FF occurred at a frequency of 57.3% in males and 32.9% in females, but this seems to be a mistake. Table 3 shows that FF occurred in 47 of 54 males (87%) and 27 of 28 females (96.4%). There is no information about the statistical test performed, but the genotype frequencies look to me to be too similar to justify the p-value of 0.02 in Table 3. There is also no information given about the multivariate analysis mentioned. Therefore, I recommend that this entire section needs to be revised, with clearer descriptions of the statistical analyses and correction of the genotype frequencies.

3. Deviation from Hardy-Weinberg Equilibrium

More details of this chi-square test are required. As far as I can tell, because the frequency of the f allele is so low, the expected number of ff samples according to HWE is zero, so I'm not sure how the chi squared test could be performed. There are methods to test for a deviation from HWE that don't have the same problem with rare alleles. For example, see Wigginton et al. (2005).

Minor concerns
1. Abstract line 1 – Mycobacterium tuberculosis is not TB, it is the causative agent of TB.

2. Introduction Par 1 - “Similarly the widely studied and functional single nucleotide polymorphism (SNP) rs2228570 of VDR fok I gene has shown variations.” The meaning of this sentence is not clear.

3. Dataset 1 only includes 35 sequences. Where are the others?

4. References missing in a few places:
- Introduction Par 1 - “Vitamin D insufficiency is common worldwide and recent studies have shown that vitamin D insufficiency is associated with a higher risk of active TB”
- Discussion Par 1 - “The ff genotype frequency of 4.8% was similar to the one described for PTB patients in the African case control study of Gambia Guinea and Guinea Bissau.”

References
Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

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