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Research letter

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Zoonotic tuberculosis in humans assessed by next-generation sequencing: an 18-month nationwide study in Lebanon

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To the Editor:

The World Health Organization (WHO) and other international organisations, including the Food and Agriculture Organization of the United Nations, the World Organisation for Animal Health and the International Union Against Tuberculosis and Lung Disease, recently called for formally assessing and (re)prioritising the burden of zoonotic tuberculosis in people, due to *Mycobacterium bovis* [1, 2]. Its global contribution to human tuberculosis, otherwise principally caused by *Mycobacterium tuberculosis*, might be underestimated [2]. Nationally representative prevalence data are virtually non-existent on continents with the highest presumed burdens, i.e. in Africa and Asia [3].

In addition to more frequently cause hard-to-diagnose extrapulmonary tuberculosis, *M. bovis* is naturally resistant to pyrazinamide [4], a crucial drug for the standard short course anti-tuberculosis therapy. Due to reliability issues, phenotypic susceptibility to pyrazinamide is often not tested [5]. The most commonly used phenotypic and molecular diagnostics, including the WHO-endorsed GeneXpert MTB/RIF test (Cepheid, USA), do not differentiate *M. bovis* from *M. tuberculosis* [1]. *M. bovis*-infected patients may thus receive inadequate treatment, risking poorer outcome [6]. Underdiagnosis in people also implies the existence of undetected animal and food sources and zoonotic risks escaping common tuberculosis control measures [1, 2].

In Europe, patients with *M. bovis* infection are often African- or Southern Mediterranean-born, suggesting regional endemicity [6, 7]. We determined the *M. bovis*-caused tuberculosis prevalence in a survey including all tuberculosis patients reported to the national tuberculosis program over an 18-month period in Lebanon. In addition to its national population and more than 1.5 million Syrian and Palestinian refugees, this Mediterranean country hosts large numbers of migrant workers from Africa and Asia [8]. Many are from Ethiopia [8], where proportions of extrapulmonary tuberculosis and *M. bovis*-caused disease among tuberculosis patients apparently culminate, reaching ~30% [9] and 15-30% (in focal studies; [10]), respectively. We used a novel targeted next-generation sequencing-based assay for extensive drug resistance detection, including to pyrazinamide, and genotypic differentiation between *M. bovis* and *M. tuberculosis* [11].

This survey was approved by the Azm Center/Lebanese University ethical committee (document CE-EDST-3-2016). Clinical samples were collected from all 1104 different TB patients with presumption of pulmonary or extrapulmonary TB, based on the presence of symptoms and prior tuberculin skin testing or radiological examination in local hospitals for a number of patients, and reported to all national anti-TB centres between June 1, 2016 and November 31, 2017. All of these 1104 were tested by at least one of the following assays: solid (Lowenstein-Jensen) and/or liquid (BBL MGIT, Beckton Dickinson, USA) culturing, GeneXpert MTB/RIF on sputum, and Anyplex MTB/NTM Real-time (Seegene, Korea) on sputum or culture on solid or liquid medium. Of these 1104, 417 were confirmed as tuberculosis positive by GeneXpert and/or Anyplex. Among these 417, 354 were culture positive. Among the 354 corresponding patients, 325 were new tuberculosis patients, 22 had a previous tuberculosis history, while tuberculosis history information was unavailable for 7 patients.

Available DNA extracts, obtained from 348/354 primary cultures by using MasterPureTM DNA Purification Kit (Epicentre, Illumina, WI, USA), were subjected to targeted sequencing, more tolerant to low DNA integrity (following suboptimal sample transportation from Lebanon to France) than whole genome sequencing. Briefly, the Deeplex-MycTB assay (Genoscreen, France) uses a 24-plexed amplification of mycobacterial species identification (*hsp*65), genotyping (spoligotyping and phylogenetic single nucleotide polymorphisms (SNPs)) and 18 *M. tuberculosis* complex drug resistance-associated gene targets, including the main pyrazinamide resistance-associated gene *pncA* (Fig. 1). Paired-end amplicon libraries of 150-bp read length (Nextera XT kit, Illumina, CA, USA) were sequenced in a single Illumina NextSeq run. Variant calling and genotypic analysis were performed using a parameterized software (GenoScreen).

Of 339/348 (97.4%) samples with exploitable sequencing data, 11 were concordantly identified as *M. bovis* strains by typical spoligotype signatures (missing spacers 39-43) and the canonical phylogenetic SNP *pncA* H57D causing natural pyrazinamide resistance [5] (Fig. 1). No other known drug resistance-associated mutations were detected. Standard 24-locus MIRU-VNTR typing [12] followed by MIRU-VNTR*Plus* database identification [13] confirmed *M. bovis* classification in all cases, and identified one additional *M. bovis* infection among the 9 samples without sequencing data. A total of

12/348 (3.4%) patients were thus infected with *M. bovis;* 11 were new tuberculosis patients. The remaining 336 had human-associated tuberculosis strains (i.e. *M. tuberculosis* or *M. africanum*).

Logistic regression analysis was used to identify independent predictors of *M. bovis* infection. Potential covariates were patient age, gender, treatment outcome, new case versus previously treated case, nationality, extrapulmonary tuberculosis. Normal distribution of selected continuous variables was assessed by the Shapiro-Wilk test. The log-linearity assumption being violated for patient age, a piecewise log-linear regression model was considered. The multivariate model was built by minimizing Schwarz's Bayesian Criterion and maximizing the c-statistics. The final model was assessed with Pearson goodness of fit test. The two-sided type I error was set at 5%. Median [25th; 75th percentile] patient age was 40 years [22; 62] with *M. bovis* vs 29 [25; 38] with human-associated strains (per year adjusted OR if age ≥55 1.14, 95% CI 1.01-1.28, p=0.04). Of the 12 patients with *M. bovis*, 5 (41.6%) had extrapulmonary tuberculosis (including 1 gastric for a two-year baby), versus 8/336 (2.4%) patients with human-associated strains (adjusted OR 16.9, 95% CI 3.5-80.7, p=0.0004). The single patient who had both pulmonary and extrapulmonary disease had a *M. tuberculosis* strain. This case was categorized as extrapulmonary when analysing the association between extrapulmonary infection and *M. bovis* or human-associated strains. While Lebanese nationals represented only 86/336 (25.6%) of the patients with human-associated strains, they comprised 10/12 (83.3%) patients with *M. bovis* (adjusted OR 6.9, 95% CI 1.4-35.5, p=0.02). The two remaining patients were a Syrian refugee and a Syrian resident. Other covariates, such as the proportion of new tuberculosis patients (92% in both *M. bovis* and human-associated strain categories), were not statistically associated with risk of *M. bovis* infection.

Patients with *M. bovis* were all from different places disseminated over the country, and unique 24-locus MIRU-VNTR types were detected for each isolate, which was unsupportive of inter-patient transmission or a common source of infection. Phylogenetic reconstruction indicated that these different strains represent at least two distinct *M. bovis* sublineages, distinguished e.g. by a *fabG1* SNP and distinct spoligotype signatures (Fig. 1). Infections by distinct strains in residents from

disseminated places inform the probable existence of multiple zoonotic sources (e.g. infected cattle, unpasteurized dairy produce) unidentified in the country.

None of the 148 migrant patients carried *M. bovis*, although they originated mainly from Ethiopia (n=94) and Sudan (n=13) where zoonotic infection has been documented or presumed and linked to widespread pastoralism [10]. Other migrants were from other high tuberculosis incidence countries (e.g. 18 from Bangladesh, 9 from The Philippines). While the lack of *M. bovis* infection among migrant workers could reflect low *M. bovis* burdens in these countries, it might also partly result from i) selective recruitment of candidates who manifest no medical issues (thus unlikely to have active tuberculosis) by overseas employment agencies in the country of origin, ii) putatively lower risk of progression to disease among patients latently infected with *M. bovis* than with *M. tuberculosis*, due to lower virulence of *M. bovis* [14, 15], iii) residency in the country of destination often limited to few years. More studies in those countries would be needed to investigate this.

In a Dutch longitudinal study, no association was found between patient outcome and the use of standard first-line TB treatment (4-month rifampicin/isoniazid/ethambutol/pyrazinamide, 2-month rifampicin/isoniazid) instead of a regimen adopted by some countries for treating *M. bovis* disease (9-month isoniazid/rifampicin, 2-month ethambutol) [6]. Nevertheless, the mortality with *M. bovis* disease was found to be higher relative to *M. tuberculosis*, presumably reflecting more prevalent miliary and central nervous system (CNS) localization of *M. bovis.* While the 12 *M. bovis*-infected patients in our observational study received the standard first-line TB treatment, none of them had a miliary or CNS localization and they were all reported as cured or with a completed treatment according to WHO classification. Prolonged follow-up will be necessary to assess their risks of relapse.

Although this first extended, nationwide estimation in a non-high income country revealed a relatively low zoonosis prevalence among tuberculosis patients (3.4%), our findings are aligned with global calls for appropriate diagnostics and treatment of these patients and tuberculosis control measures at the animal/human interface [1, 2].

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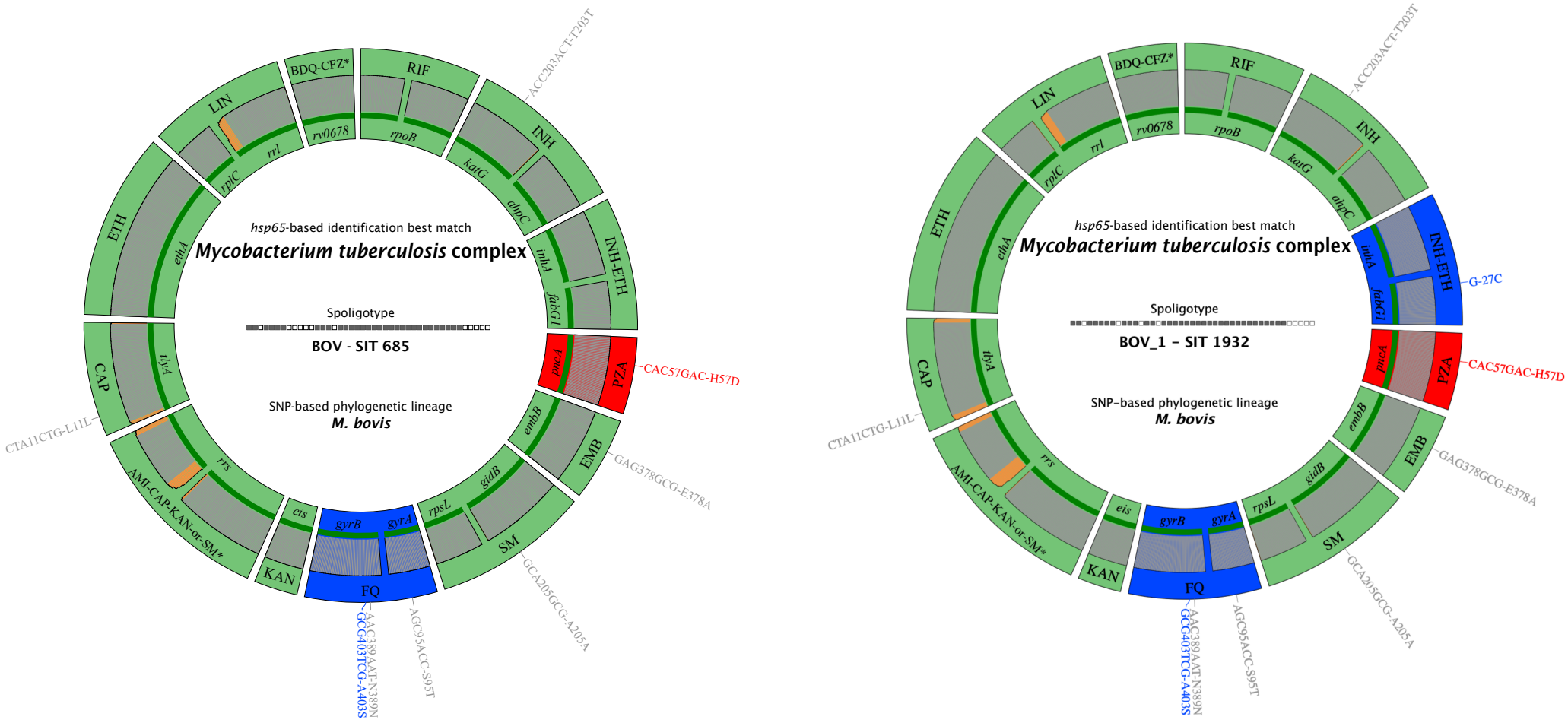

Figure legend: Deeplex-MycTB results identifying *M. bovis*-caused zoonotic tuberculosis in patients in Lebanon. Target gene regions are grouped within sectors in a circular map according to the tuberculous drug resistance with which they are associated. The sectors in red refers to the *pncA* region in which the pyrazinamide resistance-causing H57D mutation (shown in red around the circular map) canonically associated with *M. bovis* is detected. Sectors in green or blue refer to regions where no mutation or only mutations not associated with resistance (shown in gray around the map), or as yet uncharacterized mutations (shown in blue around the map) are detected, respectively. Results shown on the left and right parts correspond to two isolates representative of two strain lineages distinguished by the absence (left; comprising 7 isolates in total) or presence (right; 5 isolates in total) of the *fabG1* G-27C SNP, distinct *M. bovis* spoligotype and/or MIRU-VNTR signatures, respectively. Green lines above gene names represent the reference sequences with coverage breadth above 95%. Limits of detection (LOD) of potential heteroresistance (reflected by subpopulations of reads bearing a mutation), depending on the coverage depths over individual sequence positions, are indicated by gray (LOD 3%) and orange zones (variable LOD >3%–80%) above the reference sequences. Information on mycobacterial species identification, based on *hsp65* sequence best match, and genotype of *Mycobacterium tuberculosis* complex strain, based on spoligotype and lineage-defining, phylogenetic SNP, are shown in the center of the circle. *AMI, amikacin; BDQ, bedaquiline; CAP, capreomycin; CFZ, clofazimine; EMB, ethambutol; ETH, ethionamide; FQ, fluoroquinolones; KAN, kanamycin; LIN, linezolid; INH, isoniazid; PZA, pyrazinamide; RIF, rifampin; SM, streptomycin; SIT, spoligotype international type.