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Is *Mycobacterium africanum* Subtype II (Uganda I and Uganda II) a Genetically Well-Defined Subspecies of the *Mycobacterium tuberculosis* Complex?

In a retrospective study of the population structure of *Mycobacterium tuberculosis* complex strains from a single hospital in Kampala, Uganda, Niemann et al. (6) reported that *M. africanum* subtype II is a major cause of human tuberculosis in this area. The reclassification of strains previously isolated during the period during 1995 to 1997 as *M. africanum* subtype II relied on phenotypic characteristics such as dysgonic colony morphology, change of color of bromocresol medium, resistance of the strains to 1 μg of thiophen-2-carboxylic acid hydrazide (TCH) per ml, and growth on Lebek medium. Molecular markers such as *gyrB* single-nucleotide polymorphism, spoligotyping, and IS*6110* restriction fragment length polymorphism (RFLP) were also used to further study these isolates. According to the authors, the subtype II isolates differed from the classical *M. africanum* subtype I isolates (including the type strain ATCC 25420) by the absence of spacers 33 to 36 and 40. Additional absence of spacer 43 further subdivided the so-called "subtype II" isolates into Uganda II (only spacer 40 missing) and Uganda I (both spacers 40 and 43 missing). This paper is an interesting description of the population structure of the *M. tuberculosis* complex isolates from Uganda. However, the classification of these isolates as *M. africanum* variants, based essentially on variable phenotypic characteristics, is in contradiction with the recent findings on the evolution and phylogenetic structure of the *M. tuberculosis* complex.

The *M. tuberculosis* complex encompasses five members or variants—*M. tuberculosis*, *M. africanum*, *M. bovis*, *M. canettii*, and *M. microti.* Contrary to the other variants of the complex, *M. africanum* isolates have substantial phenotypic heterogeneity that appears to fill the phenetic discontinuity between two major pathogens: *M. bovis* and *M. tuberculosis* (3). The members of the *M. africanum* species were initially subdivided by David et al. (3) into two major subgroups, which corresponded to their geographic origin and biochemical properties. Those from western Africa, or group G of David et al., also called "subtype I," were further subdivided into G1, G2, and G3 (closer to *M. bovis*). The ones from eastern Africa, or group F of David et al., also called "subtype II," were further subdivided into F1, F2, and F3 (closer to *M. tuberculosis*).

Biochemical characters can give discordant results among laboratories if the laboratories use different criteria for interpretation, such as for growth in the presence of TCH with critical concentrations ranging from 1 to 5 μg/ml according to the laboratories. Another example is the test used by the authors to determine oxygen preference, which included Lebek medium. The interpretation of this test was not described (6), but referred back to an article from the same laboratory (5): "Aerophilic growth is indicated by growth on the surface and above the surface on the glass wall of the tube, whereas microaerophilic growth is indicated by growth below the surface." Based on this interpretation, the authors described 100% of the subtype II isolates from Uganda as microaerophilic. These results are in contradiction with previous results of David et al. (3) when using Lebek medium for F2 and F3 subclusters of *M. africanum* from Rwanda and Burundi (also eastern Africa). A more widely used medium today for such studies is the Middlebrook 7H9 broth with 0.1 to 0.2% agarose (11): "Aerobic strains grow at or near the surface, while microaerophilic strains grow as a band 10 to 20 mm below the surface, sometimes extending upwards." An example of these results clearly differentiating aerophilic and microaerophilic growth can be found in a recent publication (7). Perhaps the authors should retest the putative *M. africanum* subtype II by using this currently recommended method.

Moreover, misidentification might also arise for strains with atypical features due to multidrug resistance, a frequent finding in African countries. Consequently differentiation of *M. africanum* should no longer be based on phenotypic characteristics exclusively (10). Many strains phenotypically classified as *M. africanum* probably include not only true *M. africanum*, but also other subgroups within the *M. tuberculosis* complex whose taxonomic position is yet uncertain, and only a finer genetic analysis may help classify them correctly. Although the formal genetic definition of *M. africanum* strains remains to be established, recent papers have shed light on their phylogenetic position within the *M. tuberculosis* complex. The first paper by Streevatsan et al. (9) defined three major genetic groups (I, II, and III) relying on the $katG^{463}$-$gyrA^{95}$ single-nucleotide polymorphisms. According to this well-established and widely used scheme, the terms *M. microti*, *M. africanum*, and *M. bovis* should be reserved for *M. tuberculosis* complex group I organisms, whereas *M. tuberculosis* may be found in groups I, II, and III (9).

Based on the distribution of 20 variable regions in the genome of the *M. tuberculosis* complex, it was shown recently that *M. africanum* strains lack a specific region, RD9, and sometimes RD10 (2, 7), each of which is present in *M. tuberculosis.* *M. africanum* is also characterized by the presence of the region TbD1, a deletion specific for "modern" *M. tuberculosis* isolates (2). In a study by Brosch et al. (2), two strains from Uganda that were previously identified by phenotypic characters as *M. africanum* harbored this specific RD9 region, lacked TbD1, and presented a $katG^{463}$ CGG (Arg) corresponding to group II or III of Sreevatsan and colleagues. Results from a second study (7) confirmed the presence of the RD9 region and also demonstrated a preference for aerophilic conditions in these two strains. These results suggest that these so-called *M. africanum* strains should be regarded as *M. tuberculosis* rather than *M. africanum*. After the publication of the paper by Niemann et al. (6), we reexamined three additional strains from East Africa (two from Burundi and one from Rwanda) identified as *M. africanum* phenotypically that also bore a spoligotype signature identical to the *M. africanum* subtype II strain (Uganda I) described by Niemann et al. (5). All of these three strains did carry the RD9 region, a feature suggesting that they were not a true *M. africanum* variant. These data are in agreement with the fact that Niemann et al. (6) could not distinguish the *M. africanum* subtype II from *M. tuberculosis* on the basis of the *gyrB* single-nucleotide polymorphism, which was used by the same authors to differentiate between the different members of the *M. tuberculosis* complex.

A last point concerns the use of the missing spacer 40 (and the absence of spacers 33 to 36) as a marker that may help to designate a tubercle bacillus originating from East Africa as *M. africanum* subtype II (6). The observation that spacer 40 is absent in many strains, whatever their geographical origin, was

pointed out recently by Bifani et al. (1) for the Beijing group of *M. tuberculosis*. Besides, no strain showing simultaneous absence of spacers 33 to 36 and 40 and belonging to major genetic group I were reported in the Houston study (although many *M. africanum* subtype I strains with simultaneous absence of spacers 8 to 9 and 39 were reported in reference 8). Furthermore, we have independently observed in the Institut Pasteur de Guadeloupe spoligotype database a trend that the absence of this spacer 40 may be linked to an African origin of *M. tuberculosis*. (Reference 4 gives a current update with more than 21,000 isolates and 1,250 shared types from 100 countries.) However, spacer 40 was also missing from some other yet poorly defined clades, such as T2 and LAM4 (4; unpublished observations), which are all *M. tuberculosis* strains. Indeed, homoplasic events, due to a topological instability of this specific direct repeat (DR40), could be the cause for the frequent and independent loss of this spacer in different strain lineages. Consequently, putting too much emphasis on the absence of spacer 40 found in isolates from a single hospital in a single town of a single country and trying to generalize this observation to the dimension of an entire continent (Africa) in terms of prevalence may not be justified. Finally, it should be mentioned that in two independent studies using large sample sizes (2, 8), strains that lacked spacers 33 to 36, but had the flanking spacers present, exclusively carried the *katG*^{463} mutation CGG characteristic for *M. tuberculosis* strains of genetic group II or III. The absence of these spacers from the *M. africanum* subtype II strains described by Niemann et al. (6) represents an additional argument that these strains belong to *M. tuberculosis* group II or III strains rather than to *M. africanum*.

Based on several independent genetic markers, it appears that this interesting predominant group of strains in Uganda described as *M. africanum* subtype II phylogenetically represents a variant of *M. tuberculosis* that is distant from *M. africanum*. It seems plausible that the phenotypic similarities to *M. africanum*, such as the dysgonic colony morphology, may be due to yet-unidentified genetic changes that may have occurred independently in various members of the *M. tuberculosis* complex.

As a consequence, we therefore suggest that for future studies of *M. africanum*, the presence or absence of RD9 and the determination of the Sreevatsan's major genetic groups should be taken in consideration, before new isolates are designated as *M. africanum*. Based on these recommendations, a European network of experts is at present working to develop a consensus definition of *M. africanum* under the EU Concerted Action project QLK2-CT-2000-00630. Readers who wish to help contribute to this project may contact the undersigned investigators.

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Authors' Reply

In response to the letter by Sola et al. commenting on our recent publication (11), we would like to make the following remarks. In general, we agree that the accumulation of new findings in this important field of mycobacteriology might in future lead to revisions of the present classification of the *M. tuberculosis* complex. However, we do not fully agree with the argumentation presented by Sola et al.

In our publication, we systematically analyzed the population structure of clinical isolates from Uganda (East Africa) by phenotypical and genotypical procedures. The clinical isolates were collected at the Mulago University Hospital, which is the major referral center for patients with tuberculosis from much of the metropolitan area of Kampala, Uganda's capital. The species differentiation and denomination of the isolates were performed according to currently accepted standards and include the validly described species of the *M. tuberculosis*

complex (e.g., *DSMZ Bacterial Nomenclature Up-to-Date*," http://www.dsmz.de/bactnom/bactname.htm). The initial identification and species classification of the clinical isolates in our study were based on phenotypic characteristics. For example, *M. tuberculosis* was identified by eugonic growth on Löwenstein-Jensen slants and aerophilic growth, while identification of *M. africanum* was based on dysgonic growth on Löwenstein-Jensen slants, microaerophilic growth, or no change of color on bromcresol medium. These conventional methods have been described to be discriminative for *M. africanum* and have been used as "gold standard" methods in several previous publications (3, 6, 7, 15, 17, 18). According to their origin in East Africa and the presence of typical biochemical characteristics, the *M. africanum* strains in our study were characterized as *M. africanum* subtype II (3, 7, 9). After the initial phenotypical classification, all isolates were analyzed by spoligotyping and IS*6110* restriction fragment length polymorphism (RFLP) typing. One of the major findings in our publication is that an association could be demonstrated between two well-defined genotypes and the particular phenotypic properties of *M. africanum* subtype II. To the best of our knowledge, this is the first publication in this field with such a clear-cut observation. We further demonstrate that strains of the *M. africanum* subtype II genotypes are the major cause of tuberculosis in the area of Kampala, Uganda.

In their letter, Sola et al. state that "biochemical characters can give discordant results among laboratories if they use different criteria for interpretation" and raise concern regarding the accuracy of Lebek medium for determining the oxygen preference of *M. tuberculosis* complex isolates. Biochemical tests have long been the lone procedures for mycobacterial differentiation and at present are still among the gold standard methods for species differentiation in routine laboratories. We concede that slight variations may be observed among different laboratories; however, biochemical tests are usually performed according to national and international guidelines. Moreover, it is only a matter of good laboratory practice to include appropriate internal and external quality controls in such biochemical tests, which then ensures the accuracy of the tests performed. Our laboratory serves as a national and supranational reference laboratory and thus participates twice a year in external proficiency testing (both national and international). For all test panels, negative and positive internal controls were included. We are confident that on this basis, reliable and reproducible data were generated in our laboratory that can withstand international comparison.

As concerns the Lebek semisolid medium; this medium has been used routinely since about 1960, is a well-accepted growth medium for analysis of the *M. tuberculosis* complex, and was the basis for differentiation between *M. tuberculosis* and *M. africanum* in several previous studies (6, 7, 13) in addition to the study by David et al. (3). Our laboratory has at least 30 years of experience with this method. Furthermore, Lebek medium is recommended by the official German guidelines for differentiation of the *M. tuberculosis* complex (4). To the best of our knowledge, comparable published experience and evidence regarding the accuracy of the 7H9 broth-based version of this method for differentiation of the *M. tuberculosis* complex do not exist.

The argument "multidrug resistance, a frequent finding in African countries" appears misleading, if not wrong. It is understood that resistance to isoniazid interferes with the test for TCH resistance and that both simultaneous results for isoniazid and TCH resistance therefore must be available. However, high levels of multidrug resistance have not been reported from many African countries so far (5). In our strain collection from Uganda ($n = 234$), only one *M. tuberculosis* isolate and three *M. africanum i*solates were resistant to isoniazid and thus cross-resistant to TCH. Moreover, the species differentiation in our study (11) was not based on a single test, which might have resulted in misidentification, but on a combination of test results, which were evaluated in combination to achieve an accurate strain differentiation (see Table 1 in our paper).

We believe that the main findings of the publications that were cited to support Sola's discussion of molecular characteristics of *M. africanum* (1, 12, 14) might be biased by the following two crucial points: (i) only small numbers of *M. africanum* strains were analyzed, and (ii) the majority of isolates were from West African countries. The studies presented by Sreevatsan et al. (14), Brosch et al. (1), and Parsons et al. (12) together included only about 60 *M. africanum* isolates. These studies nicely demonstrated the existence of a particular *M. africanum* genotype in West Africa, which is characterized by the absence of RD9 and particular mutations in *katG-gyrA*^{95}. This West African *M. africanum* genotype (subtype I, resembling *M. bovis*) has also been confirmed by characteristic IS*6110* fingerprint and spoligotype features (intermediate or small number of IS*6110* bands, spoligotype pattern intermediate between *M. bovis* and *M. tuberculosis*) in studies performed by others (7, 8, 16) and those performed by us (9, 10). Our results further indicated that the West African subtype can be identified easily by a specific single-nucleotide polymorphism in the *gyrB* gene (10).

Evidence for a particular genotype associated with phenotypic characteristics of *M. africanum* subtype II (resembling *M. tuberculosis*) in East Africa have been found in a study performed by Haas et al. (7) and in our previous study (9). The existence of this genotype has been confirmed in our present study, which systematically analyzed the population structure of a large sample of *M. tuberculosis* complex isolates from Kampala, Uganda (East Africa) (11). Whether or not RD9 is generally present or absent in these isolates remains unclear. Whether the RD9 deletion might be useful as a differentiation marker cannot be answered by the analysis of just five isolates from Uganda as suggested by Sola et al.

With regards to Sola's concern of the use of spacer 40 as a differentiation criterion for the two *M. africanum* subtype II families, we intended to point out that absence of spacer 40 is a frequent characteristic of both genotypes, but it can also be found occasionally in *M. tuberculosis* isolates. We do not suggest that absence of spacer 40 is the unique and specific criterion for the identification of *M. africanum* subtype II and want to point out the identification of the genotypes in our recent study (11) based on the IS*6110* DNA fingerprint analysis. However, considering our database of spoligotypes of German *M. tuberculosis* isolates, spacer 40 is present in the great majority of *M. tuberculosis* isolates, and its absence thus may serve in combination with other characteristics, such as phenotypic characteristics, as an identification tool for *M. africanum* subtype II.

Altogether, data presented in previous studies related to *M. africanum* confirm the existence of two *M. africanum* genotype families in Africa: one associated with subtype I phenotypic characteristics (West Africa) and one associated with subtype II phenotypic characteristics (East Africa, Uganda). Molecular typing results did not really yield new insights into the phylogenetics of *M. africanum*. To the contrary, molecular typing methods so far have only confirmed the earlier classifications, proposed on the basis of numerical analyses of phenotypic characteristics (3). There is no justification for Sola's claim that

M. africanum strains from West Africa are the sole "true" *M. africanum* strains and that strains from East Africa should be considered as non-*M. africanum* or *M. tuberculosis* subtypes. The species description of *M. africanum* based on phenotypic characteristics included both subtypes, one closely related to *M. bovis* and one closely related to *M. tuberculosis*, and this has now been confirmed by molecular data. The importance of the RD9 deletion in this context requires further evaluation based on representative data from East African strains.

In conclusion, we believe that *M. africanum* subtype I and *M. africanum* subtype II represent two unique phylogenetic branches within the *M. tuberculosis* complex that originate in West and East Africa, respectively. Prospective studies of large numbers of strains, however, will be needed for more detailed analyses of the regional prevalence and molecular characteristics of *M. africanum*. Should a new species description of *M. africanum* including use of new molecular markers be intended, larger studies comprising representative numbers of clinical strains from West and East Africa would be warranted. Finally, we are well aware of the work of the EU Concerted Action project QLK-CT-2000-00632. We are confident that a consensus definition of *M. africanum* soon will be developed by the working group of this project, which was initiated with our participation at the Concerted Action meeting in May 2002 after a long discussion of *M. africanum* differentiation.

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