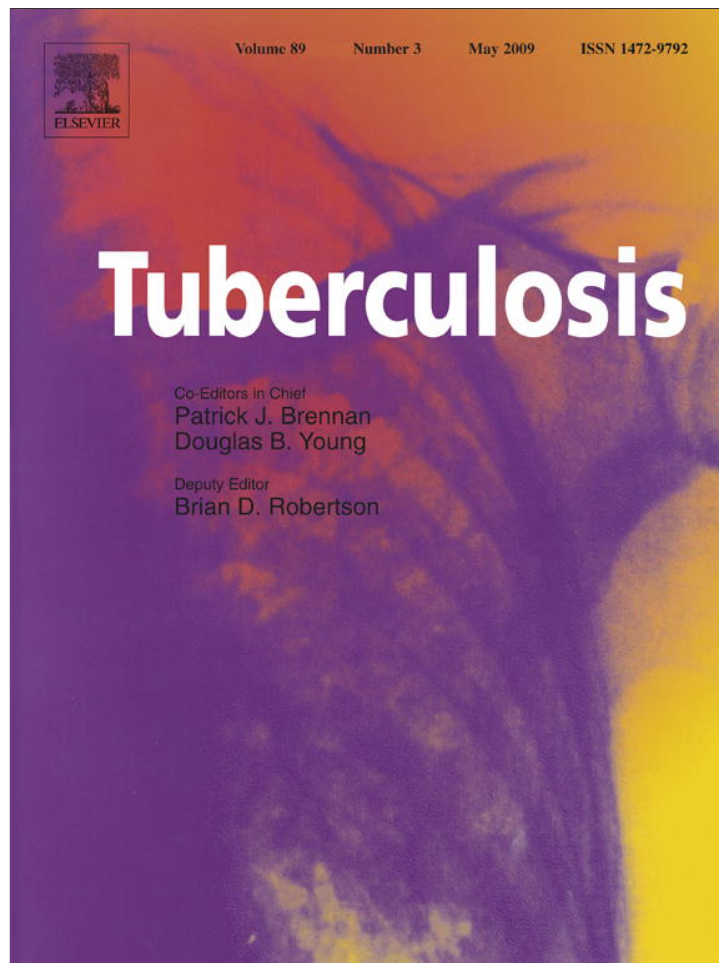


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## MOLECULAR ASPECTS

## Comparison of four-colour IS6110-fAFLP with the classic IS6110-RFLP on the ability to detect recent transmission in the city of Barcelona, Spain

Sònia Borrell<sup>a</sup>, Nicola Thorne<sup>b</sup>, Montserrat Español<sup>c</sup>, Chloe Mortimer<sup>b</sup>, Àngels Orcau<sup>d</sup>, Pere Coll<sup>c,e</sup>, Saheer Gharbia<sup>b</sup>, Julian González-Martín<sup>a</sup>, Catherine Arnold<sup>b,\*</sup><sup>a</sup>S. de Microbiología, CDB, H. Clínico de Barcelona-IDIBAPS, Universidad de Barcelona, Spain<sup>b</sup>Applied and Functional Genomics, Centre for Infections, Health Protection Agency, 61 Colindale Avenue, London NW9 5EQ, UK<sup>c</sup>S. de Microbiología, H.U. Sant Pau, Barcelona, Spain<sup>d</sup>Servei d'Epidemiologia, Agència de Salut Pública de Barcelona, CIBER de Epidemiologia y Salud Pública (CIBERESP), Spain<sup>e</sup>Servei de Microbiologia, Hospital de la Santa Creu i Sant Pau, Departament de Genètica i Microbiologia, Universitat Autònoma de Barcelona, Spain

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## SUMMARY

The purpose of the study was to compare the IS6110-RFLP (RFLP) results obtained in a previous epidemiological study in the city of Barcelona, Spain [Borrell S, Espanol M, Orcau A, Tudo G, March F, Cayla JA, et al. Factors associated with differences between conventional contact tracing and molecular epidemiology in study of tuberculosis transmission and analysis in the city of Barcelona, Spain. *J Clin Microbiol* 2009 Jan;47(1):198–204.] with the results obtained with IS6110-fAFLP, [Thorne N, Evans JT, Smith EG, Hawkey PM, Gharbia S, Arnold C. An IS6110-targeting fluorescent amplified fragment length polymorphism alternative to IS6110-restriction fragment length polymorphism analysis for *Mycobacterium tuberculosis* DNA fingerprinting. *Clin Microbiol Infect* 2007 Oct;13(10):964–70.] on the ability to detect recent transmission. fAFLP was applied to DNA samples of RFLP clustered strains, with and without known epidemiological links, with the additional inclusion of four nucleotide-specific fluorophores to further increase the discrimination of the fragments obtained.

Four-colour fAFLP was performed on 123 RFLP clustered strains with no epidemiological link (NELC) and on 28 epidemiologically linked RFLP clustered (ELC) strains grouped into 48 and 13 clusters respectively. Clustering results obtained by the two methods were highly congruent in ELC strains with fAFLP allocating 92.3% of the ELCs. For the NELCs, RFLP results were confirmed in 39/48 (81.2%) of fAFLP-clusters with 0–1 different fragments and 9/48 (18.8%) differed in 2–4 fragments, which are considered genetically related but not recently transmitted.

In conclusion, overestimation of recent tuberculosis transmission can occur because of the inaccurate analysis of RFLP results. Four-colour fAFLP allows us to differentiate between recent transmission strains and epidemiologically unrelated but genetically related strains.

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## 1. Introduction

IS6110-restriction fragment length polymorphism (RFLP)<sup>1</sup> has been the reference technique of choice for genotyping *Mycobacterium tuberculosis* since 1993. The high degree of IS6110 polymorphism, both in copy number and position, between different *M. tuberculosis* strains, has made it a useful marker for strain genotyping. IS6110 transposition events are generally common enough

to allow differentiation between unrelated strains but are still rare enough to show stability within more closely related strains,<sup>1,2</sup> making it useful in distinguishing between distant and recent transmission of epidemiological events.

Since the technique was standardized, RFLP has been extensively applied to analyse the amount of recent transmission comparing the results with conventional epidemiological information in different contexts and populations.<sup>3–6</sup> However, the need for well-grown cultures and purified DNA taking 4–6 weeks to produce results, in addition to sometimes subjective analysis of RFLP patterns and subsequent overestimation of recent transmission, can result in a low correlation between the molecular and the conventional epidemiology.

\* Corresponding author. Tel.: +44 (0)20 8327 6068; fax: +44 (0)20 8327 6738.  
E-mail address: [catherine.arnold@hpa.org.uk](mailto:catherine.arnold@hpa.org.uk) (C. Arnold).

Therefore more accurate techniques should be developed to differentiate extensive transmission or outbreaks rapidly and efficiently from what appear to be sporadic, epidemiologically unrelated episodes. This is especially important regarding clustered isolates with an unknown link, which in the literature has been mainly attributed to unsuspected recent transmission,<sup>3,7</sup> noting the possibility of sporadic contacts and indicating that Conventional Contact Tracing procedures should be directed to improve the search for these cases. However a proportion of these clusters could be related to TB reactivation caused by prevalent strains<sup>8,9</sup> or to the phenomenon of convergent evolution.<sup>10</sup>

In that way recently, different PCR-based techniques as alternatives to RFLP have been developed<sup>11–14</sup> but study of IS6110 copy number and genomic insertion point using a PCR-based method is still not widely used.

In 2007 Thorne et al. published<sup>15</sup> a fluorescent amplified fragment length polymorphism fingerprinting method that mixed the potential of PCR typing with the superior resolution of the IS6110 marker. IS6110-fAFLP (fAFLP) detects precisely sized fragments which enable rapid and accurate interpretation and, importantly, comparison of large numbers of strains. This exact sizing of fragments within the fAFLP typing profiles enables a more informed and rapid decision to be made based on genotyping data alone.

Data results from a previous epidemiological study in the city of Barcelona, Spain from 2003 to 2004<sup>16</sup> described RFLP clustered strains that had unknown epidemiological relationships. To try to resolve the real amount of recent transmission, RFLP data obtained in Barcelona were compared with the results obtained with fAFLP<sup>15</sup> to test the clinical application of fAFLP on describing recent transmission and to measure the discriminatory power of RFLP on clustering non-epidemiological linked strains.

## 2. Materials and methods

### 2.1. Strain selection

The selected DNAs were from the strain collection described in Borrell et al. 2009<sup>16</sup> and comprised of 123 RFLP clustered strains with no epidemiological link (NELCs) and 28 epidemiologically linked RFLP clustered strains (ELCs) grouped into 48 and 13 clusters respectively. 500 ng of DNA used for the IS6110-RFLP<sup>1</sup> and extracted in the Hospital Clinic de Barcelona and in the Hospital de Santa Creu i Sant Pau, Barcelona, Spain was sent as an unidentified panel to the Health Protection Agency Centre for Infection, London, UK. All clustered strains had an RFLP pattern with six or more bands. The number of bands in the RFLP patterns ranged from 5 to 14 bands. In the patterns with 6 or less than 6 RFLP bands, MIRU12 was applied as a second marker, as described in Ref. 16.

### 2.2. Principal genetic grouping

Principal Genetic Group (PGG) assignment<sup>17</sup> was carried out for all strains according to the methods described by Sreevatsan et al.

### 2.3. IS6110-fAFLP typing

IS6110-targetting fAFLP methodology was performed as previously described,<sup>15</sup> with the modification of including four differentially labelled TaqI adaptor primers instead of one primer, each with one of the four selective bases A, C, G and T at the 3' end for increased resolution with the following dyes and sequences (all 5'–3'): Taq1\_G = VIC-CGATGAGTCCTGACCGAG; Taq1\_A = 6-FAM-CGATGAGTCCTGACCGAA; Taq1\_T = NED-CGATGAGTCCTGACCGAT; Taq1\_C = PET-CGATGAGTCCTGACCGAC. The fragments were separated on an ABI 3130xl, (Applied Biosystems, Warrington, Cheshire,

UK) with 0.4 ul of LIZ-600 size standard and analysed in GeneMapper v4.0 (Applied Biosystems). Fragments were considered identical if they were same 'colour' and were within 0.5 base pairs of each other.

### 2.4. Automated fragment size analysis

Automated sizing of fluorescently labelled fragments was performed using the Applied Biosystems 3130xl using the LIZ-600 size standard.

### 2.5. Cluster analysis

An fAFLP-cluster was defined as isolates with an identical fAFLP profile, with identical size and colour fragments, or with a single fragment difference.

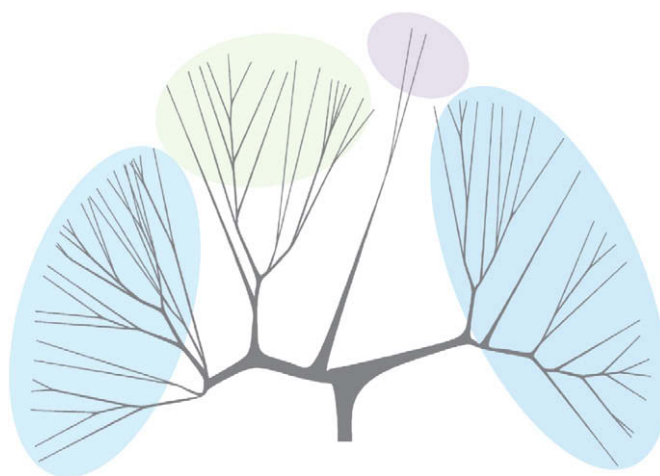
To determine the phylogenetic signal of the fAFLP, fragments identified in GeneMapper were entered as character data (i.e. the presence or absence of a fragment of a specific size and colour) into BioNumerics version 4.5 (Applied Maths, Saint-Martens-Latem, Belgium). A tree was created using the DICE coefficient and UPGMA settings. The tree was rooted and displayed as a rendered type (Figures 1 and 2).

## 3. Results

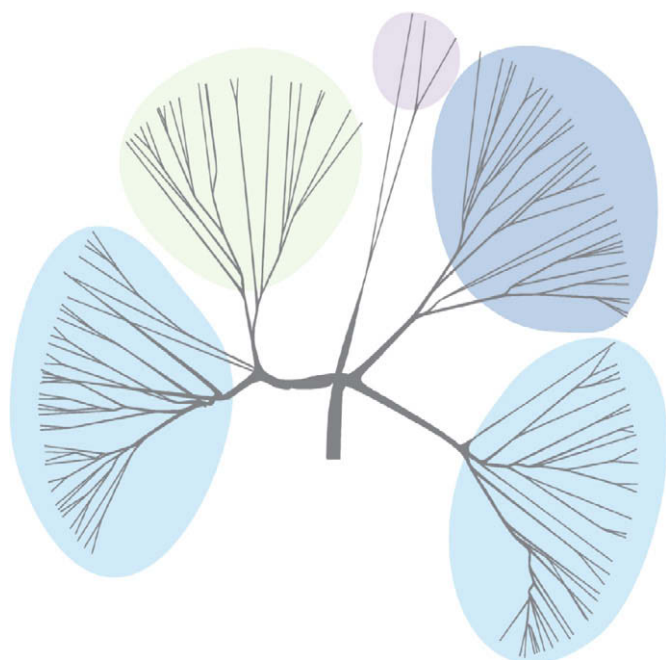
Rendered trees were obtained for both techniques (Figures 1 and 2) with all tested strains, including ELCs and NELCs, and showed similar topology, suggesting a high correlation of the two data types. Nevertheless a further, separate branch, is present in the fAFLP tree but not in the RFLP tree, showing a higher discriminatory power of fAFLP. Different tree building methods were used to generate trees, including Neighbour Joining, and gave the same or similar topology, suggesting that the trees and their clustering are robust.

### 3.1. ELC strains

ELCs had epidemiological link confirmed by Conventional Contact Tracing and were grouped by RFLP in 13 clusters. Clustering results obtained by the two methods were highly congruent. Following fAFLP-cluster definition, fAFLP allocated 12/13 (92.3%) of the ELCs in exactly the same cluster (0–1 fragment different). In 77%



**Figure 1.** IS6110-RFLP rendered tree generated using BioNumerics and colour coded into genetic lineages. Principal Genetic Group (PGG) 1 is coloured pink, PGG2 is coloured blue and PGG3 is coloured green.



**Figure 2.** IS6110-fAFLP rendered tree generated using BioNumerics and colour coded into genetic lineages. PGG1 is coloured pink, PGG2 is coloured blue and PGG3 is coloured green. Although the identical strains used to generate Figure 1 are included and fall into similar groupings using both techniques, the PGG2 group on the right hand side of Figure 1 is now split into two distinct groups using this methodology, indicating increased resolution.

(10/13) of the ELCs, the strains involved in the fAFLP-cluster had exactly the same number of fragments (size and colour) and in the three (23%) remaining fAFLP-clusters one strain had one fragment difference. The number of fAFLP fragments obtained were equal (7/13), less (3/13) or more (3/13) in comparison with the number of RFLP bands.

One of the ELCs clusters (1/13) was a school outbreak of three members; RFLP grouped the three strains in the same cluster and joined another strain which no epidemiological relation found (Figure 3). When fAFLP was applied to the four samples, the three students shared an identical profile, while in the fourth sample (11238) two different fragments were shown confirming no epidemiologically detected recent transmission. Although those four strains might have been related in some time point of the past (13 fragments shared) the two new IS6110 copies show the more evolved profile of the fourth case.

### 3.2. NELC strains

NELCs had no epidemiological link confirmed by Conventional Contact Tracing and were grouped by RFLP in 48 RFLP-clusters. The majority of the NELC clusters were composed of two (29/48) and three (14/48) members with no epidemiological link between them.

RFLP results were confirmed in 39/48 (81.2%) of fAFLP-clusters with 0–1 different fragments, 9/48 (18.8%) differed by two fragments, which are considered genetically related but not recently transmitted and one differed by four fragments. The RFLP cluster with four different fAFLP fragments was a cluster of two strains with a pattern of eight RFLP bands. The fAFLP profile showed eight common but four different fragments discarding the recent transmission.

The number of fAFLP fragments obtained were equal (21/39), less (7/39) or more (11/39) in comparison with the number of RFLP bands of the confirmed clusters.

## 4. Discussion

The present study attempts to correlate the results of the well known gold standard TB genotyping technique; IS6110-RFLP<sup>1</sup> with the IS6110-fAFLP<sup>15</sup> described by Thorne et al. in 2007. In both techniques the molecular marker used is the same, so a high correlation of results and a similar percentage of clustering are expected.

Although this high correlation was already described in Thorne et al., some technical differences were added in the current study to improve the discriminatory power of the fAFLP.

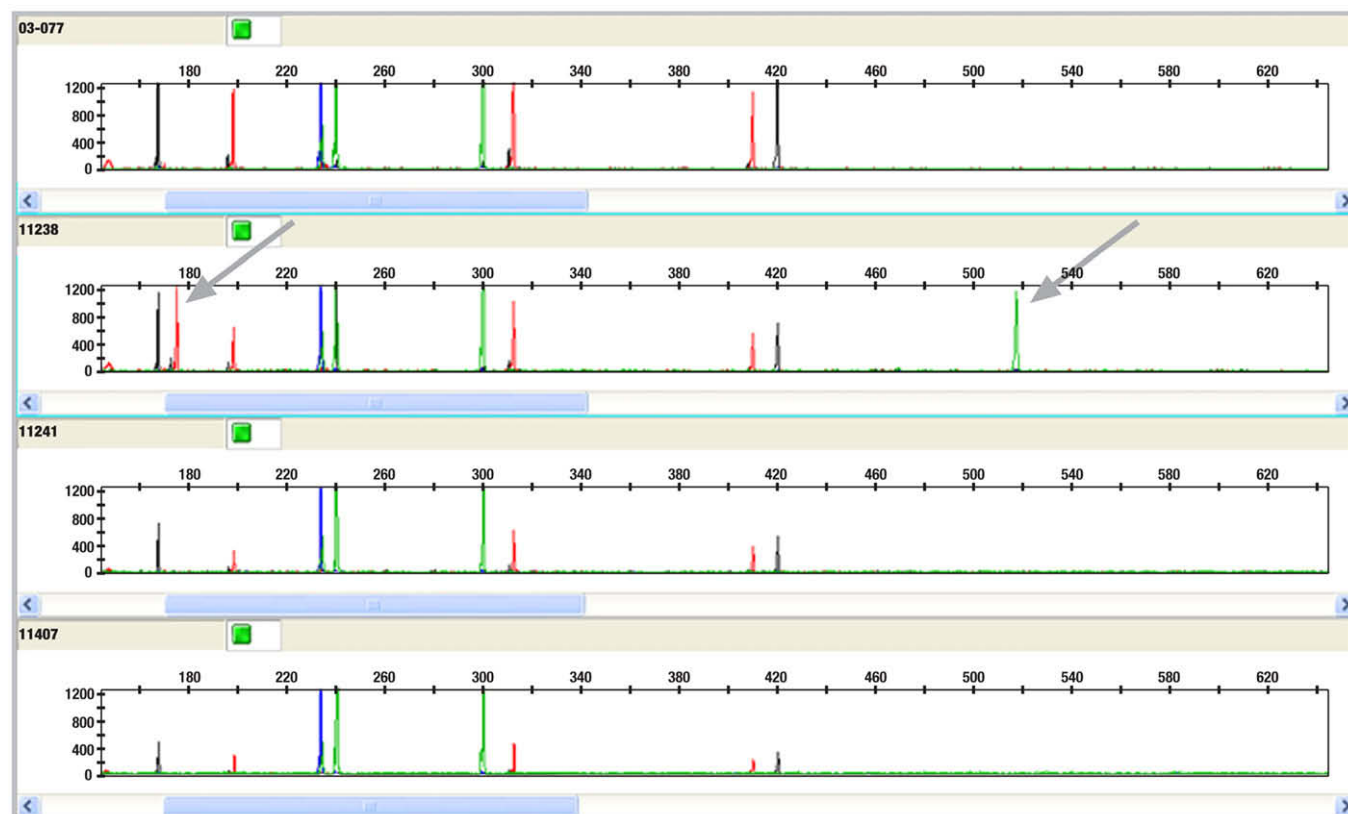
fAFLP automatically detects the IS6110 fragments produced after a digestion and a ligation protocol, permitting a simpler and accurate detection of the exact number of IS6110 instead of the subjective analysis of IS6110 bands in RFLP radiography. The novel technical incorporation included the addition of four differentially labelled primers instead of one, each with one of the four selective bases at the 3' end for increased resolution. This change in the original technique allows the differentiation between overlapping similarly sized fragments, which will now most likely be of a different colour and facilitate the analysis of the fAFLP profiles.

In Figures 1 and 2 the high correlation is shown by the similar topology of the displayed trees. Nevertheless a further, separate branch, is present in the fAFLP tree but not in the RFLP tree, showing a higher evolutionary discriminatory power of fAFLP.<sup>17</sup>

One important limitation of the classic RFLP is that the resolution afforded is lower for low copy strains,<sup>2,18,19</sup> such that identical hybridization patterns may not indicate clonality when six or fewer bands co-migrate. Although an IS6110-probed band on a hybridization blot indicates the presence and size of the PvuII–PvuII IS6110-associated DNA fragment, it does not provide the chromosomal location of the IS element. Therefore identical bands may be from distinct genomic locales.<sup>19</sup> Because fAFLP gives a specific size and colour of the fragments, apparently identical RFLP bands might not share the same nucleotide beside the enzyme cutting site, thereafter the apparently identical RFLP fragments will be of different colour by fAFLP.

To test the correlation between clinical samples of known epidemiology, fAFLP was applied to epidemiological related (ELC) and unrelated strains (NELCs). Clustering results in the ELC group of strains were highly congruent (92.3%). In 23% of the ELCs, one fragment difference was observed. Avoiding misinterpretation of low intensity bands<sup>20</sup> and migration differences in RFLP clustered strains with 1 RFLP band difference has been extensively described in the literature<sup>21,22</sup> as in recently transmitted strains. The automatic detection of fAFLP fragments means more precision and because of that, differences of one fAFLP fragment in confirmed recent transmission strains should be interpreted as being associated with more rapid evolution of the IS6110 in certain situations,<sup>21,23–25</sup> such as being inserted into active transcriptional insertion sites<sup>2</sup> or to undergo higher selective pressures, possibly dependent on strain-specific *in vivo* replication rates, host–pathogen interaction or anatomical properties.<sup>26,27</sup>

Sixteen percent of the NELCs differed by two or more fragments excluding those strains related by recent transmission events. Although these fragment differences indicated that these strains were not recently related, they still shared between 8 and 13 fAFLP fragments, suggesting evolution from the same ancestral clone in the past. This is evidenced in the school outbreak shown in Figure 3,

**RFLP-IS6110****fAFLP-IS6110**

**Figure 3.** School outbreak. RFLP patterns and fAFLP profiles showing the specific different fluorescent fragments indicated by an arrow. The screen shot showing fAFLP profiles shows a window of 160–620 base pairs for clarity. Other smaller bands are present below 160 base pairs and the total number correlates to the number of RFLP bands. The extra RFLP band shown in the 11407 RFLP pattern was not present in the fAFLP profile.

illustrating the importance of the precision of fAFLP results for appropriate correlation with conventional contact study information.

Because the fAFLP technique is based on the same marker as RFLP, the number of fAFLP fragments expected should be the same as the number of RFLP bands, which occurred in the majority of the clusters analysed. In some cases the number of fragments can be less than the number of bands because the marker used for the automatic detection only analyse fragments within a certain size (70–600 bp). However, few fragments are expected to be smaller than 70 bp or larger than 600 bp.

Conversely, when more fAFLP fragments than RFLP bands are detected, it might be due to the presence of, sometimes not counted, low intensity bands in the RFLP radiograph<sup>19</sup> or because of the difficult task of differentiation between one or two bands were the migration distance of two bands appears to be identical.

In addition to the previously described advantages of the technique, fAFLP analysis is a high-throughput, accurate, precise and technically simple method. The amount of DNA needed is 500 ng and it is possible to process up to 96 samples, from DNA extract to

final fAFLP profile, in less than 3 days with approximately three hours 'hands-on' time.

In conclusion, overestimation of recent tuberculosis transmission can occur because of the inaccurate analysis of RFLP results. A more precise technique to detect the number and position of *IS6110* copies is needed to improve this. Four-colour fAFLP, with an automated detection and analysis system, is likely to allow greater differentiation between recently transmitted strains and epidemiologically unrelated but genetically related strains more precisely. The precision and automation of the technique allow complete portability between laboratories and less ambiguous interpretation of results.

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**Competing interests:** None declared.

**Ethical approval:** Not required.

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