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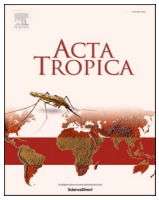

Coinfection of a yaws patient with two closely related *Treponema pallidum* subsp. *pertenue* strains: A rare event with potential evolutionary implications

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ABSTRACT

The etiological agent of yaws is the spirochete *Treponema pallidum* (TP) subsp. *pertenue* (TPE) and infects the children of Papua New Guinea, causing ulcerative skin lesions that impairs normal growth and development. Closely related strains of *Treponema pallidum* subsp. *pertenue,* J_E11, and T_E13 were detected in an ulcer biospecimen derived from a 5-year-old yaws patient. Cloning experiments validated the presence of two distinct but similar genotypes, namely T_E13 and J_E11, co-occurring within a single host. While coinfection with highly related TPE strains has only limited epidemiological and clinical relevance, this is the first documented coinfection with genetically distinct TP strains in a single patient. Similar coinfections in the past were explained by the existence of over a dozen recombinant loci present in the TP genomes as a result of inter-strain or inter-subspecies recombination events following an anticipated scenario of TP coinfection, i.e., uptake of foreign DNA and DNA recombination.

1. Introduction

Yaws is an endemic tropical disease caused by *Treponema pallidum* (TP) subsp. *pertenue* (TPE) affecting mostly children in impoverished regions of the tropics (Kline et al., 2013). Clinical manifestations of yaws include chronic erythematous lesions or ulcers predominantly on the lower limbs, and when left untreated, can advance to secondary and tertiary yaws affecting the skin and bones (Giacani. and Lukehart, 2014; Marks et al., 2018).

Molecular typing of TPE isolates performed on swab samples obtained from young patients in Namatanai, Papua New Guinea (PNG), during a clinical trial conducted from 2018 and 2019 (John et al., 2022), found 255 fully typed TPE-positive samples among the 1,081 tested swab samples. A multilocus sequence analysis of 3 typing loci (TP0548, TP0488, and TP0858) revealed three allelic profiles (AP) (J_E11, T_E13, and S_E22) among the PNG clinical isolates (Medappa et al., 2024). The most predominant strain circulating in Namatanai was J_E11 (93.5 %), followed by S_E22 (4.3 %), and T_E13 (2.2 %) (Medappa et al., 2024).

Out of the 255 fully typed TPE-positive samples, a single sample revealed the presence of two infecting TPE genotypes (Medappa et al., 2024). Coinfection of a single patient with distinct TP genotypes, to our best knowledge, has never been described in patients infected with TPE or related treponemes.

Coinfection is likely prerequisite for inter-strain and inter-clade recombinations as observed in syphilis clinical isolates from the Americas and Europe (Grillová et al., 2019a, 2019b; Kumar et al., 2018; Lieberman et al., 2022) and in syphilis reference strains (Grillová et al., 2019b; Kumar et al., 2018; Pětrošová et al., 2012).

Here, we present cloning experiments validating the presence of the two distinct genotypes, T_E13 and J_E11, co-occurring in a single sample taken from a single host (a five-year-old male patient from Bungbuwe, Namatanai, PNG)

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2. Material and methods

2.1. Patient sample

The patient sample came from a swab of a skin ulcer on a five-year-old male residing in Bungbuwe, Namatanai, Papua New Guinea. The sample was collected from an exudative ulcer on the lower limb and stored in a lysis buffer (100 mM TRIS, 100 mM EDTA, 1 % SDS, pH = 8) at −20 °C. DNA isolated from the original swab was tested for genome equivalent number of treponemal DNA with quantitative PCR targeting the *pol*A gene using primers Tpa_polA_F (5′- GAGTGTGCAGTCCG CTATGC-3′) and Tpa_polA_R (5′-AGGCAAAAGCGGCATTTCTA-3′) that amplified a 129 bp-long partial sequence of the *pol*A gene, which was detected using the Tpa_polA_P (5′-6FAM-TCCGCTTGGAAACAGCAGG ATTG-BHQ1-3′) probe (Dubourg et al., 2015). The procedure was performed using a qPCR master mix QuantiFast® Probe PCR +ROX Vial Kit from Qiagen (Hilden, Germany). Quantification was performed by extrapolation of standard curves using serial dilutions of vector pCR2.1-TOPO (Invitrogen, Carlsbad, CA, USA) that contained cloned PCR products produced by Tpa_polA_F and Tpa_polA_R primers. The original ratio of the J_E11 and T_E13 genotypes in the co-infection sample was determined using DNA cloning and sequencing.

2.2. DNA isolation and multilocus sequence typing (MLST)

Genomic DNA was extracted using a QIAamp DNA mini kit according to the manufacturer's instructions (Qiagen). TPE positivity was assessed using PCR amplification of *pol*A (TP0105, Cruz et al., 2010). Multilocus sequence typing (MLST) was performed using four typing targets: TP0326, TP0548, TP0858, and TP0865. Regions between coordinates 347098–348277 (1179 bp), 593246–594263 (1018 bp), 936044–937019 (976 bp), and 944543–946833 (2291) were screened for genetic variability (coordinates based on TPE Samoa D genome, Čejková et al., 2012, CP002374) for the TP0326, TP0548, TP0858, and TP0865 genes, respectively. Nested PCR was performed using the following cycling conditions: 1st step with PrimeSTAR GXL DNA polymerase (Takara Bio Inc., Otsu, Japan): 94 °C initial denaturation for 1 min, 8 cycles at 98 °C (10 s), 68 °C (15 s) with Touch down of −1 °C per cycle and 68 °C (1 m 45 s), 35 cycles at 98 °C (10 s), 61 °C (15 s) and 68 °C (1 m 45 s), and final extension at 68 °C (7 m). PCR amplifications for the 1st step were performed in a total volume of 25 μl consisting of 5× PrimeSTAR GXL buffer, PrimeSTAR dNTP mix, and PrimeSTAR GXL DNA polymerase, The 2nd step was carried out using *Taq* polymerase (New England Biolabs, Frankfurt am Main, Germany): 94 °C initial denaturation for one minute, 40 cycles at 94 °C (30 s), 48 °C (30 s), 72 °C (1 m 45 s) and final extension at 72 °C for 7 min. PCR amplifications were performed in a total volume of 25 μl with 1 μl of template from the first step of the reaction. The master mix included 10× Standard *Taq* reaction buffer, 100 mM dNTPs diluted to give a final concentration of 200 μM, and *Taq* DNA polymerase. The master mix was for a 25 μl reaction as per the manufacturer's protocol. MLST primers were described previously (John et al., 2022). PCR products were sequenced using Sanger sequencing at GATC Biotech AG (Eurofins Genomics Company, Constance, Germany). Sequencing analyses were performed using the Lasergene program package (DNASTAR v.7.1.0; DNASTAR, Madison, WI, USA).

2.3. DNA cloning

DNA was amplified using nested PCR with PrimeSTAR GXL DNA polymerase used for both steps. The products of the second step were treated with *Taq* polymerase (0.2 μl of *Taq* per reaction, 10 min at 72 °C; New England Biolabs) to introduce adenine overhangs at their 3′ ends for TA cloning. The PCR products were cloned into a pCR2.1-TOPO vector (Invitrogen, Waltham, MA, USA) according to the manufacturer's instructions. Following cloning, transformation into competent *E. coli* DH10B cells was performed using heat shock transformation. Colony PCR using *Taq* polymerase was employed for screening clones with the insert. Cycling conditions for colony PCR were as follows: 94 °C initial denaturation for 5 min, 40 cycles at 94 °C (1 m), 55 °C (1 m), 72 °C (1 m 30 s), and final extension at 72 °C (7 m).

2.4. In vitro simulation of PCR amplification

Yaws clinical isolates that were exclusively J_E11 (T19.ba.25) and T_E13 (T19.ba.2) were PCR amplified at the TP0858 locus. TP0858 amplification included two steps of nested PCR as described above; PrimeSTAR GXL DNA polymerase was used for both steps. Following PCR amplification, cloning, and transformation as described above were performed individually for each clinical isolate. Screened clones having inserts were then Sanger sequenced. One clone each from J_E11 and T_E13 was selected and sequenced, and the two clones were then combined in a 1:3 ratio (i.e., J_E11:T_E13 ratio = 1:3); the copy number was similar to that found in the sample from the coinfected patient (i.e., 10,000 copies μl^{-1}) determined by the detection of the *pol*A count. Subsequently, PCR amplification, cloning, and transformation protocols were performed as described above. A set of 96 clones was sequenced.

3. Results

3.1. Origin of sample from coinfected patient

A single patient, a five-year-old male residing in Bungbuwe village (Upper Sentral Niu Ailan rural local level government area, SNA region, Fig. 1), Namatanai, PNG, was found to be coinfected with two TPE strains of different allelic profiles (AP), i.e., J_E11 and T_E13 (Medappa et al., 2024). The coinfected individual was treated in Ward 11, which contained 10 participants in total, out of which five were positive when tested for TPE DNA (J_E11 ($n = 3$), T_E13 ($n = 1$), and J_E11/T_E13 ($n = 1$)). Ward 11 comprises 8 villages (Bungbuwe, Dalom, Damon, Kadan, Kantubu, Konangusngus, Lamerika Pt, and Livinko). Patients positive for J_E11 were from Livinko and Konangusngus. Patients infected with T_E13 (sample no. T04.ba.81) and the patient coinfected with J_E11/T_E13 (sample no. T04.ba.79) came from Bungbuwe (Latitude: −3.23348, Longitude: 151.99779).

As shown in Fig. 1, positions of individual wards in the vicinity of Ward 11 are indicated together with identified TPE genotypes in individual wards. Ward 11 (with J_E11 ($n = 3$), T_E13 ($n = 1$), and J_E11/T_E13 ($n = 1$) genotypes) is surrounded by wards with patients mostly infected with J_E11. Blue and red dots denote villages in SNA and NTI local-level government areas, respectively. This map was constructed using QGIS 3.28.3 Firenze; the base layer was made using Natural Earth. Free vector and raster map @naturalearthdata.com.

3.2. Analysis of the sample from coinfected patient

The coinfection was confirmed by the detection of two distinct genotypes (allelic profiles) by cloning PCR products from four genomic loci, TP0326, TP0548, TP0858, and TP0865 (Table 1). Both TPE allelic profiles J_E11 and T_E13 differed in the TP0326, TP0548, TP0858, and TP0865 loci in 7, 10, 32, and 14 nucleotide positions, respectively. Clones containing amplified PCR products of TP0326 ($n = 23$), TP0548 ($n = 8$), TP0858 ($n = 96$), and TP0865 ($n = 33$) consistently revealed coinfection with a predominance of T_E13 allelic profile compared to the J_E11 profile. Interestingly, several cloned PCR products resembled molecular chimeras (Table 1). A total of 11 chimeric patterns were found (Table 2); the four main chimeric patterns are shown in Fig. 2. Altogether, based on analysis of 4 genomic loci and 160 individual clones, the original sample contained two distinct types of alleles (corresponding to allelic profiles J_E11 and T_E13) and 21.3 % of recombinant alleles (Table 1), which is consistent with existence of two distinct TPE genotypes. Presence of two sequentially related TPE genotypes could,

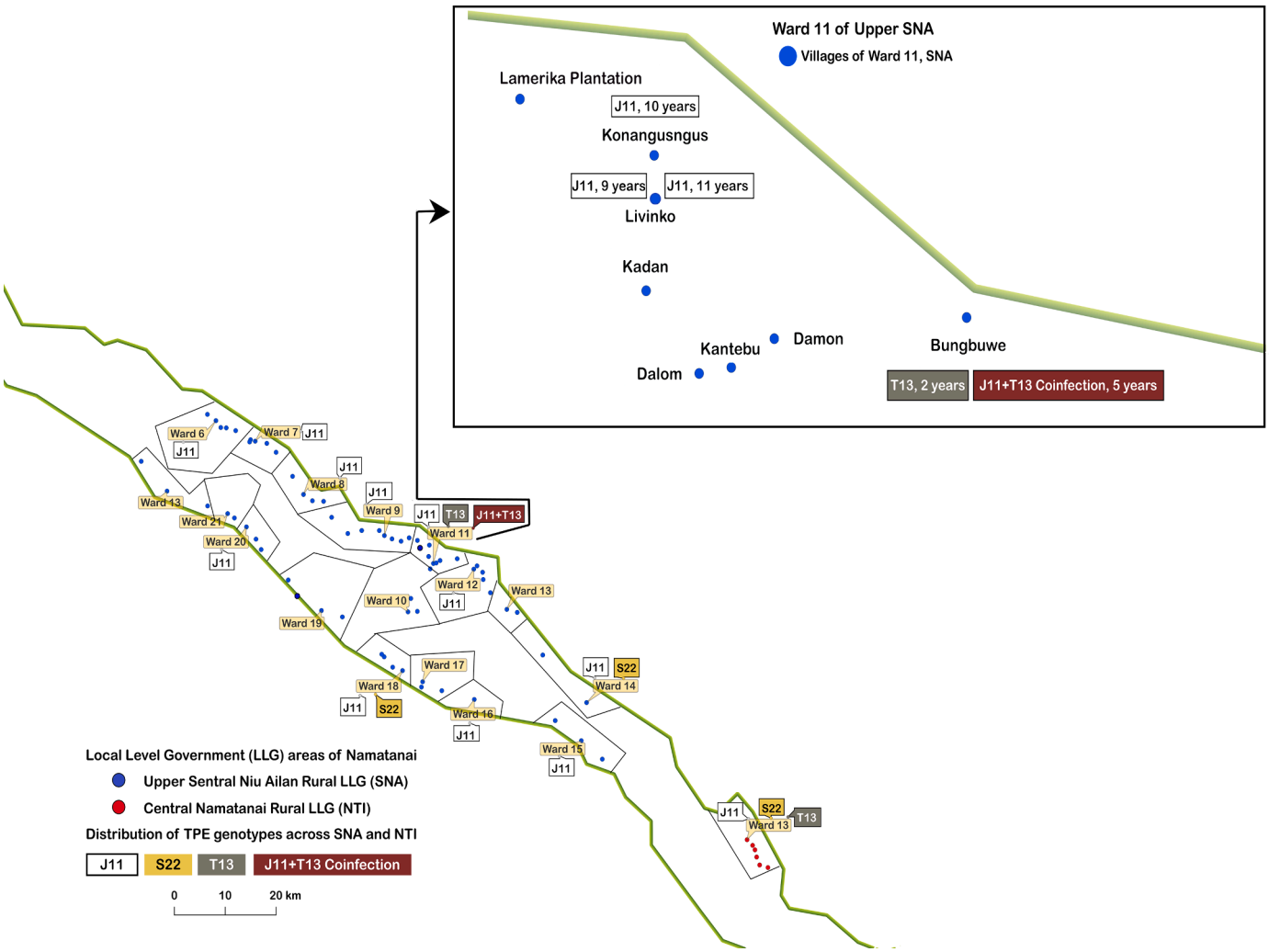

Fig. 1. Positions of individual wards in the vicinity of Ward 11 and identified TPE genotypes in individual wards.

Table 1
Allelic variants were determined for each tested locus. A total of 160 clones were screened.

Number of clones screened per locus	J_E11 (%)	T_E13 (%)	PCR chimeras (%)	T_E13/ J_E11
TP0326 ($n = 23$)	1 (4.3)	16 (69.6)	6 (26.1)	16/1
TP0548 ($n = 8$)	2 (25.0)	3 (37.5)	3 (37.5)	3/2
TP0858 ($n = 96$)	17 (17.7)	63 (65.6)	16 (16.7)	63/17
TP0865 ($n = 33$)	2 (6.1)	22 (66.7)	9 (27.2)	22/2

during PCR amplification, artificially produce molecular chimeras resembling recombinant alleles.

3.3. Analysis of PCR product chimeras

To assess whether the cloned PCR product chimeras were due to a chimeric template or due to chimeras emerging during PCR amplification, reamplification of the TP0858 locus was performed with a mixture of pure target DNA of both J_E11 and T_E13 in the same ratio to mimic the same conditions as in the original coinfected sample. Under these conditions, analysis of the TP0858 clones ($n = 96$) revealed the presence of PCR chimeras ($n = 9$), although in a somewhat reduced prevalence (9.4 %) compared to chimeras detected among the cloned PCR products taken directly from the sample (34 chimeras from 160 clones, 21.3 %). With the exception of chimeric pattern 1, which was not found among the clones from purified target DNA, similar chimeric patterns were detected among cloned PCR products taken directly from the clinical sample and from purified target DNA (Fig. 2, Table 2). Moreover, chimeric patterns 2 and 3 were found to be more frequent compared to chimeric patterns 1 and 4 under both conditions (Table 2).

Table 2
Chimeric patterns were found among cloned PCR products from clinical samples and purified target DNA mixtures.

Chimeric pattern	PCR clones ($n = 16$) from the original sample (%)	PCR clones ($n = 9$) from a mixture of purified target DNA (%)
1	2 (12.5)	0
2	6 (37.5)	3 (33.3)
3	3 (18.75)	2 (22.2)
4	1 (6.25)	1 (11.1)
5	1 (6.25)	0
6	1 (6.25)	0
7	1 (6.25)	0
8	1 (6.25)	0
9	0	1 (11.1)
10	0	1 (11.1)
11	0	1 (11.1)

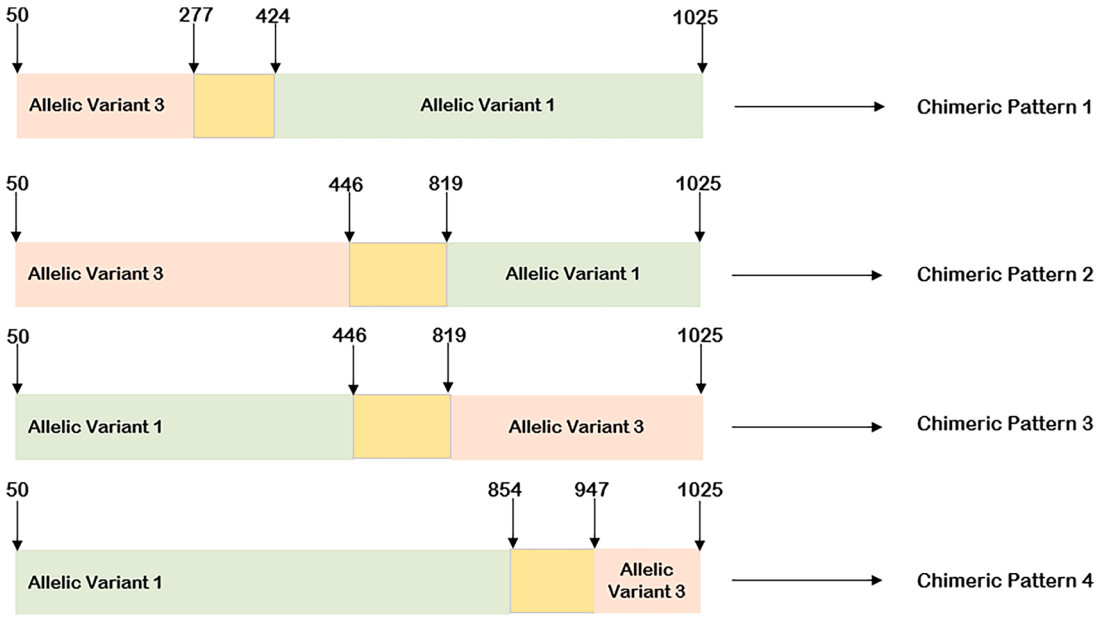

Fig. 2. Frequent chimeric patterns ($n = 4$) and the corresponding breakpoints (sequences were identical for both alleles) as observed among cloned PCR products from the clinical sample and purified target DNA. The coordinate positions refer to the TP0858 gene of TPE Samoa D. Yellow regions represent gene sequences (with whole genome coordinates 936271–936418, 936440–936813, and 936848–936941 for chimeric pattern 1, chimeric pattern 2-3, and chimeric pattern 4, respectively) where the transition from one sequence type to the other sequence type has occurred. Chimeric pattern 1 was observed only in PCR products from the clinical sample, whereas chimeric patterns 2, 3, and 4 were observed in PCR products prepared from both target DNA preparations.

4. Discussion

A single clinical sample (a skin ulcer swab) containing two different TPE allelic profiles, J_E11 and T_E13, taken from a 5-year-old child from the Bungbuwe village of Namatanai, PNG (Medappa et al., 2024), was analyzed in this study. To date and to our knowledge, there are no verified reports showing coinfection with genetically different molecular types among patients infected with TPA, TPE, or TEN.

Since the analyzed ulcer swab sample contained both genotypes in almost equimolar ratios (J_E11: T_E13 ratio of 1:3), and since TPE treponemes cannot multiply outside a primate host, this finding is consistent with coinfection of a single host with two different TPE molecular types. This prediction is further supported by the fact that both J_E11 and T_E13 allelic profiles were found among TPE isolates in the investigated population (and from patients in the same ward). The majority of infected patients in the Namatanai region, PNG, were infected with the TPE J_E11 genotype (93.5 %), and only a minority of patients were infected with T_E13 (2.2 %) (Medappa et al., 2024). Therefore, while infection with the TPE J_E11 genotype was expected, there was one patient in Ward 11 of SNA (sample no. T04.ba.81) infected with the T_E13 TPE genotype, suggesting that this patient could be the source of the T_E13 infection in the coinfected patient.

Although artificial cross-contamination of the clinical sample cannot be completely ruled out, there are several pieces of evidence supporting that this was not the case. The original sample (T04.ba.79) arrived at the laboratory with a single swab inside the transport tube, precluding contamination from another swab(s). In addition, the swab was taken from the subject in Bungbuwe during the baseline visit, and from this village, only one additional sample was positive for the T_E13 genotype, while samples positive for the J_E11 genotype were found in different villages (Livinko and Konangusngus) of Ward 11, making contamination less probable due to sequential collection of samples from each village. Moreover, subsequent contamination in the laboratory can be excluded since repeated analyses of the original sample (still in the form of the original single swab) revealed both J_E11 and T_E13 genotypes in the sample. In addition, indications of sample contamination were not found following a review of patient records and a direct correspondence with the team supervisor overseeing Ward 11 of Sentral Niu Ailan.

Cloning of PCR products and the subsequent sequencing of individual clones proved that there was a combination of two different genotypes (J_E11 and T_E13) in the original sample. Interestingly, about 21 % of analyzed clones were chimeric, combining sequences from both J_E11 and T_E13 genotypes into a single sequence. Previous studies on the amplification of highly similar molecular targets estimated the number of chimeric molecules to be between 12.9 and 30 % (Meyerhans et al., 1990; Wang and Wang, 1996), depending on sequence target similarity, number of cycles, and the ratio of the different target molecules. To verify or exclude the presence of recombinant treponemal loci (for a list of the yet-to-be-identified TP recombinant loci see Noda et al., 2022; Pla-Díaz et al., 2022), we cloned PCR products from four independent loci and found both non-recombinant and chimeric molecules in all of them, indicating PCR artifacts rather than the presence of recombinant loci. Moreover, PCR artifacts were verified by their artificial reproduction using two purified target molecules, further indicating that these chimeric sequences were the results of PCR and did not represent recombinant treponemal loci. In addition, we noticed four common breakpoints for the chimeric clones that were localized between coordinates 227–424, 446–819, and 854–947 (gene coordinates according to TPE Samoa D), where the transition from one sequence type to another sequence type occurred. Although most screened clones had a single breakpoint, we observed two breakpoints in one clone. The occurrence of more than one breakpoint, i.e., up to eight breakpoints per sequence, has been previously reported (Lahr and Katz, 2009).

The coinfection of a single human subject with two genetically distinct TPA, TPE, or TEN strains appears to be a very rare event that has never previously been clearly demonstrated in the literature. In animals, two different TPE strains were thought to infect three baboons (Chuma et al., 2019), although the infecting strains were found to be different at only a single locus. Two different TPA allelic profiles were determined in a single patient in a study of 393 genotyped human samples (Sweeney et al., 2022), suggesting a potential coinfection with two different TPA strains. Similarly, a study by Fernández-Naval et al. (2019) identified two sequentially different TPA genotypes of TP0548 (types ‘d’ and ‘g’) in the ulcer and in the blood specimens from a single patient, which may be

an example of another coinfection case. However, no further verifications were performed in the abovementioned cases. An additional patient in the Fernández-Naval et al., 2019 study had TP0548 variant types 'f' and 'g' in ulcer and blood samples; however, the 'f' and 'g' variants of TP0548 differ in only two nucleotide replacements; therefore, this case could represent intrastrain heterogeneity at this locus, as suggested by the authors (Fernández-Naval et al., 2019). While intrastrain heterogeneity can mimic coinfection with two different strains, it is usually limited to a single or only a few nucleotide positions and is also limited to specific genetic loci (Mikalová et al., 2013; Čejková et al., 2015; Šmajs et al., 2018). There are numerous examples of genetic heterogeneity in TP that include the *tpr* genes (Stamm and Bergen, 2000; Centurion-Lara et al., 2000; LaFond et al., 2003; Mikalová et al., 2020); the donor site of the *tpr* gene is close to TP0126 (Šmajs et al., 2002) and other genomic loci including TP0341, TP0402, TP0967, TP0971, and TP1029 (Matějková et al., 2008; Pětrošová et al., 2013). Altogether, genetic heterogeneity in treponemes has been found in more than 70 treponemal genes (reviewed in Šmajs et al., 2018). However, the main difference between coinfection and intrastrain heterogeneity is the presence of consistent genetic differences observed at several loci that match new or known sequence allelic variants. In this study, the data are fully consistent with patient coinfection with two different TPE strain types (i.e., allelic profiles J_E11 and T_E13).

While coinfection of a single human with two genetically distinct TP strains appears to have limited epidemiological and clinical relevance, its evolutionary potential is of significant importance since it has been previously postulated that coinfection with different TP subspecies is a prerequisite for the observed TP inter-subspecies recombination events (Pětrošová et al., 2012; Štaudová et al., 2014; Mikalová et al., 2017). Coinfections of a single human with genetically distinct treponemes (that are not able to replicate without a host and have no plasmids or phages) can explain the existence of over a dozen individual recombination events (Noda et al., 2022; Pla-Díaz et al., 2022; Maděránková et al., 2019); it also assumes that treponemes have the natural competence to take up foreign, yet related DNA. Inter-strain and inter-clade recombination events have been observed in several genes including those encoding predicted outer membrane proteins (Grillová et al., 2019b; Pětrošová et al., 2012) and were detected also in clinical samples from Cuba, France, and the Czech Republic (Grillová et al., 2019b). Furthermore, recombination was observed in several *tpr* gene positions encoding surface-exposed beta-barrels, in clinical samples from Cali (Colombia), San Francisco (California) and the Czech Republic (Kumar et al., 2018). Moreover, another study suggested occurrence of inter-strain recombinations in clinical isolates in the *arp* gene (TP0433) (Lieberman et al., 2022).

According to this scenario, coinfection is followed by a natural DNA transformation with related but sequentially distinct DNA and subsequent recombination of this DNA into chromosomal loci. As shown by remnants of gene conversions or reciprocal translocations, these recombination mechanisms are present within TP cells (Gray et al., 2006; Čejková et al., 2013) and could be responsible for the final step in a recombination event. Since treponemal pathogens are known to lack effective horizontal DNA transfer mechanisms, coinfection (including uptake of foreign DNA) and genome recombination could, therefore, represent an important mechanism in the evolution of treponemal genomes.

CRediT authorship contribution statement

Monica Medappa: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation. **Petra Pospíšilová:** Writing – review & editing, Validation, Supervision, Methodology, Investigation. **Lucy N. John:** Investigation, Data curation. **Camila González-Beiras:** Investigation, Data curation. **Oriol Mitjà:** Supervision, Resources, Project administration, Investigation, Data curation, Conceptualization. **David Šmajs:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The manuscript contains all relevant data.

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