Tetratrichomonads from the oral cavity and respiratory tract of humans

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SUMMARY

To clarify the taxonomy of trichomonads associated with human respiratory diseases, we examined a collection of axenic trichomonad strains isolated from the oral cavity and bronchi of patients from pulmonary diseases clinics in Tallin, Estonia. The oral and bronchial strains were compared mutually as well as with a reference strain of *Trichomonas tenax*, a common inhabitant of the human oral cavity, and other trichomonad species from humans and animals. Unexpectedly, the morphological studies, as well as DNA sequencing of ITS1-5.8S rRNA-ITS2 regions revealed that the Estonian strains belong to the genus *Tetratrichomonas*, with a high similarity to the avian species *Tetratrichomonas gallinarum*. None of the strains belonged to *Trichomonas tenax*. DNA fingerprinting using the RAPD method separated Estonian strains into 2 distinct groups: 'bronchial' consisting of 5 and 2 strains isolated from bronchi and 'oral' cavity, respectively, and oral consisting of 3 oral strains. Consistent differences between 'bronchial' and 'oral' groups were confirmed by analysis of ITS1-5.8S rRNA-ITS2 sequences. Our results have revealed novel trichomonad species of the human oral cavity and bronchi.

Key words: *Trichomonas tenax*, *Tetratrichomonas* sp., human, respiratory tract, oral cavity, ITS1-5.8S rRNA-ITS2, RAPD, morphology.

INTRODUCTION

The common parabasalid flagellate *Trichomonas tenax* frequently inhabits the oral cavity of humans. It has a world-wide distribution with high prevalence in persons with periodontal diseases (reviewed by Honigberg and Burgess, 1994), although the contribution of *T. tenax* to pathological changes in the periodontium is debated (Feki *et al.* 1981; Honigberg, 1990). More importantly, a number of cases of pulmonary trichomoniasis ascribed to *T. tenax* have been reported (reviewed by Hersh, 1985 and Honigberg, 1990). Besides broncho-pulmonary exudates the trichomonads have also been found in pleural fluid of predisposed patients (Memik, 1968; Walzer, Rutherford and East, 1978; Osborne, Giltman and Uthman, 1984; Ohkura, Suzuki and Hashiguchi, 1985; Shiota *et al.* 1998; Porcheret *et al.* 2002; Lewis *et al.* 2003), submaxillary gland (Duboucher, Mogenet and Perie, 1995), and infra-auricular lymph node (Duboucher *et al.* 2000).

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The generally accepted view is that trichomonads occurring in the oral cavity and the respiratory tract of humans are *T. tenax* (Honigberg and Burgess, 1994). However, some authors considered the trichomonads found in the respiratory tract to be independent species and named them on the basis of their location *Trichomonas pulmonalis* (Schmidt, 1895) or *Trichomonas broncho-pulmonalis* (Kazakova, Roigas and Teras, 1977). In addition, reports have been published on symptomatic infections of respiratory passages and oral mucosa by *T. vaginalis* in adults (Rebhun, 1964; Terezhalmy, 1983) including a case of an immunocompromised patient infected with HIV (Duboucher *et al.* 2003), or newborns infected during delivery by mothers with trichomonad vaginitis (McLaren *et al.* 1983; Hiemstra, Van Bel and Berger, 1984). Although a method for specific detection of *T. tenax* by polymerase chain reaction is available (Kikuta *et al.* 1997), most reports of the identification of parasites, if attempted at all, have relied on microscopic examination using unreliable methods for dependable classification of trichomonad species.

Trichomonas tenax infections in patients with respiratory diseases were extensively studied in Estonia. In the prospective study by Tumka (1956), trichomonads were found in 19 of 111 patients with chronic pneumonia. Kazakova *et al.* (1980) reported *T. tenax* in the bronchi of 37 out of 370 patients with chronic

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Table 1. List of trichomonad strains

No.	Species	Strain	Host	Origin
1	*Tetratrichomonas* sp.	TXB1	Human (bronchi)	Tallin, Estonia, 1975
2	*Tetratrichomonas* sp.	TXB2	Human (bronchi)	Tallin, Estonia, 1976
3	*Tetratrichomonas* sp.	TXB3	Human (bronchi)	Tallin, Estonia, 1976
4	*Tetratrichomonas* sp.	TXB4	Human (bronchi)	Tallin, Estonia, 1977
5	*Tetratrichomonas* sp.	TXB5	Human (bronchi)	Tallin, Estonia, 1977
6	*Tetratrichomonas* sp.	TXO6	Human (oral cavity)	Tallin, Estonia, 1964
7	*Tetratrichomonas* sp.	TXO7	Human (oral cavity)	Tallin, Estonia, 1973
8	*Tetratrichomonas* sp.	TXO8	Human (oral cavity)	Tallin, Estonia, 1975
9	*Tetratrichomonas* sp.	TXO9	Human (oral cavity)	Tallin, Estonia, 1977
10	*Tetratrichomonas* sp.	TXO10	Human (oral cavity)	Tallin, Estonia, 1977
11	*Trichomonas vaginalis*	TV10-02	Human (vagina)	Prague, Czech Republic, 1973
12	*Trichomonas vaginalis*	TV5-27	Human (vagina)	Prague, Czech Republic, 1973
13	*Trichomonas vaginalis*	TV85-08	Human (vagina)	Prague, Czech Republic, 1973
14	*Trichomonas vaginalis*	FF28	Human (vagina)	Bratislava, Slovakia, 1987
15	*Trichomonas vaginalis*	SBO	Human (vagina)	Gothenburg, Sweden, 1979
16	*Trichomonas gallinae*	TGK	*Columba livia* f. *domestica* (crop)	Prague, Czech Republic, 1994
17	*Tritrichomonas foetus*	LUB	*Bos taurus* (rectum)	Lublin, Poland, 1965
18	*Pentatrichomonas hominis*	HOM-V3	Human (faeces)	Da Nang, Vietnam, 1988
19	*Trichomonas tenax*, ATTC 30207	HS-4:NIH	Human (oral cavity)	Maryland,USA, 1959
20	*Tetratrichomonas gallinarum*	ZPA2	*Anas platyrhynchos*	České Budêjovice, Czech Republic, 1985
21	*Tetratrichomonas gallinarum*	1-11-M2	*Meleagris gallopavo*	Brno, Czech Republic, 1971
22	*Tetratrichomonas gallinarum*	Z26/7	*Gallus gallus* (beak)	Brno, Czech Republic, 1972
23	*Tetratrichomonas prowazeki*	SL	*Anguis fragilis*	Vrchlabí, Czech Republic, 1996
24	*Tetratrichomonas limacis*	LMA	*Limax maximus*	Kamberk, Czech Republic, 1999
25	*Trichomonas canistomae*	BRIXI	*Canis familiaris*	Kladno, Czech Republic, 1999

pneumonia and bronchitis. Sardis *et al*. (1983) found trichomonads in the bronchi of 16 out of 30 patients with various pulmonary diseases. These investigators successfully established several axenic isolates of oral trichomonads from asymptomatic patients (Teras *et al*. 1970; Teras, Kumm and Kallas, 1972), as well as from bronchi, sputum and oral cavity of patients suffering from different chronic pulmonary diseases (Kazakova *et al*. 1980, 1985). These isolates were used in comparative serological studies involving both sera of patients with pulmonary trichomoniasis (Roigas *et al*. 1980; Kazakova *et al*. 1985) and polyclonal rabbit antibodies raised against representative isolates. The results obtained with the aid of complement fixation reaction, immunofluorescence, and agglutination revealed distinct serotypes among these organisms and showed considerable differences between the strains isolated from the bronchi and those from the oral cavity. Interestingly, antigenic differences were demonstrated even between bronchial and oral isolates obtained from a single patient. On the basis of these results the authors suggested that bronchial and oral trichomonads belonging to different serotypes may represent different subspecies (Kazakova *et al*. 1980, 1985) if not species (Kazakova *et al*. 1977).

In the present study, the oral and bronchial strains of human trichomonads isolated and axenized in Estonia by Teras and colleagues (1980) were compared mutually as well as with a *T. tenax* reference strain and other trichomonad species from humans and animals with the aim being to clarify the taxonomy of trichomonads associated with human respiratory diseases. Surprisingly, genetic analysis as well as morphological observations revealed that the archival Estonian strains, of both oral and bronchial origin, are distinct from *T. tenax* and belong to the genus *Tetratrichomonas* that has not previously been reported as a human parasite.

MATERIALS AND METHODS

Organisms and cultivation

The Estonian strains were isolated from patients suffering from different chronic pulmonary diseases at the State Antituberculosis Dispensary and the Pulmonary Disease Department, Institute of Experimental and Clinical Medicine, Tallin, Estonia between 1964 and 1977 (Teras *et al*. 1980) (Table 1). The strains TXB1–TXB5 were isolated by means of bronchoscopy, and strains TXO6–TXO10 were isolated from oral cavity or sputum. Samples containing trichomonads were initially inoculated to Locke-egg-serum double phase medium LSM-2 (Wantland, Wantland and Winquist, 1963) without antibiotics, and maintained at 37 °C. The cultures were axenized using TT1 medium (Teras *et al*. 1970) as described by Kazakova *et al*. (1985). After the transfer to the Prague laboratory, the strains were adapted to the TYM medium (Clark and Diamond,

2002), cryopreserved and stored in liquid nitrogen. Other trichomonad strains (Table 1) were grown in axenic culture except for *Trichomonas canistomae*, *Tetratrichomonas limacis* and *Tetratrichomonas prowazeki* that were cultivated xenically. The axenic strains were grown in TYM medium (Clark and Diamond, 2002) at pH 6·2 (*Trichomonas vaginalis*), or pH 7·2 (all other strains) at 37 °C. The TYSGM medium (Clark and Diamond, 2002) without mucin and with the addition of rice starch was used for xenic cultures that were maintained at 37 °C (*T. canistomae*) or 26 °C (*T. limacis*, and *T. prowazeki*). These strains are available at the Department of Parasitology, Faculty of Science, Charles University in Prague.

Light microscopy

Moist films of trichomonads were spread onto coverslips that were fixed in Hollande's fluid and stained with protargol as described previously (Tachezy *et al*. 2002).

DNA isolation

Genomic DNA was isolated from trichomonads using the method of guanidium thiocyanate or guanidium chloride extraction (Pramanick, Forstová and Pivec, 1976; Tachezy *et al*. 2002). DNA isolated from strain numbers 12–15 and 18 (see Table 1) was kindly provided by Vladimír Hampl (Charles University, Prague).

Random amplified polymorphic DNA (RAPD) analysis

Random oligonucleotides (10-mers) (RAPD Primer set #2, http://www.michaelsmith.ucb.ca/services/NAPSPrimer_Sets/Primers.pdf) were obtained from the University of British Columbia, Vancouver, Canada. Each primer (UBC157, UBC173, UBC174, UBC 175, UBC177, UBC178, UBC188, UBC190, UBC194) provided distinct patterns of amplified DNA fragments. DNA amplifications were performed in PTC-200 thermocycler (MJ Research) using the following parameters: initial denaturation at 94 °C, 5 min; 35 cycles of 40 s at 92 °C, 40 s at 32 °C, and 90 s at 72 °C; 9 min. final step at 72 °C. Reaction mixtures consisted of 50 ng DNA, 10 pmol of primer, 5 mM $MgCl_2$, 400 μM of each of dNTPs, PCR reaction buffer, and 5 units of *Taq* Polymerase (Promega) in a total volume of 50 μl. PCR products were separated in a 2% (w/v) agarose gel at 80 V for 6 h. Electrophoretic patterns were scored according to Tachezy *et al*. (2002). FreeTree program (Hampl, Pavlicek and Flegr, 2001) was used for construction of trees and for computation of bootstrap values. Genetic distances of samples were computed from Nei-Li's coefficient of similarity (Nei and Li, 1979) and the tree was constructed by the neighbor-joining method (Saitou and Nei, 1987). Bootstrap values were computed for every node of the dendrogram based on 1000 resamplings.

Amplification and cloning of internal transcribed spacer (ITS) regions and 5.8S ribosomal RNA genes

Primers and conditions for DNA amplification of the ITS1-5.8S rRNA-ITS2 region were adapted from Felleisen (1997). DNA was amplified using primers complementary to conserved regions near to the 3′-end of the small subunit of the ribosomal RNA gene (ITSR: 5′-TTC AGT TCA GCG GGT CTT CC), and at the 5′-end of the large subunit of the ribosomal RNA gene (ITSF: 5′-GTA GGT GAA CCT GCC GTT GG). The PCR products of 370–500 bp were separated in a 2% (w/v) agarose gel, extracted from the gel by means of a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany), and subcloned using an Original TA Cloning Kit (Invitrogen, Carlsbad, CA, USA). Selected clones were sequenced in both directions using T3/T7α and M13 primers. ITS regions of *T. gallinarum* strain numbers 20–22 were sequenced directly from PCR products using the ITSF and ITSR primers. The sequences of strain numbers 3, 5–9, 20–25, were submitted to GenBank (National Center of Biotechnology Information) under accession numbers: Estonian strains – AY244641 (TXB3), AY244642 (TXB5), AY244644 (TXO6), AY244645 (TXO7), AY244646 (TXO8), AY244643 (TXO9); *Tetratrichomonas gallinarum* – AY244647 (ZPA2), AY244648 (Z26/7), AY244649 (1-11-M2); *Tetratrichomonas prowazeki* AY244650; *Tetratrichomonas limacis* AY244651; and *Trichomonas canistomae* AY244652.

Sequence analysis

Trichomonad sequences of the ITS1-5.8S rRNA-ITS2 regions were obtained directly in this study or from existing sequences in GenBank (*Tetratrichomonas* sp. DS, AF236105; *Tetratrichomonas* 1998-5003, AF340154; *Tetratrichomonas* 1999-5000, AF342742; *Tetratrichomonas* 2000-0017, AF342740; *Tetratrichomonas tenax*, U86615; *Trichomonas canistomae*, AY244652; *Trichomonas gallinae*, U86614; *Trichomonas vaginalis*, U86613; *Pentatrichomonas hominis*, U86616; *Tritrichomonas foetus*, U85967; *Tritrichomonas suis*, U85966; *Tritrichomonas mobilensis*, U86612). The sequences were aligned using the ClustalX, v. 1.81 program (Thompson *et al*. 2000) and manually edited using the Bioedit program v. 5.0.0. (Hall, 1999) and MUST program v. 1.0 (Philippe, 1993). All positions containing gaps were removed from the alignment. Phylogenetic trees and distance matrix were constructed using the PHYLIP program, v. 3.6 alpha (Felsenstein, 1997). Bootstrap

analyses were performed with 1000 replicates for each method. Dendrograms were constructed using the methods of neighbor-joining, Maximum Likelihood and Maximum Parsimony.

Experimental infections

Protozoan-free turkey poults were used for experimental infections as previously described (Kulda, Suchankova and Svoboda, 1974). Three groups, each containing eight 10-day-old poults, were intercloacally infected with the Estonian strains TXB5 (bronchial) and TXO6 (oral), and by *Tetratrichomonas gallinarum* strain 1-11-M2 of turkey origin. Infection doses were $2 \times 250\,\mu$l of trichomonads (4×10^6 cells/ml) in TYM medium, pH 7·2. A control group of non-infected animals consisted of 6 poults. Each group of poults was maintained separately and fed with coccidiostat-free diet. Birds were sacrificed 7 days after inoculation and their coecal content was examined by microscopy (fresh mounts) and cultivation using TYM medium supplemented with antibiotics (penicillin 2000 I.U./ml and amicacin 300 μg/ml). Animal experiments were performed according to Animal Welfare and Animal Protection Protocols of the Ministry of Agriculture of the Czech Republic.

RESULTS

Morphology

Estonian strains isolated either from the respiratory tract (strains TXB1–TXB5) or from the oral cavity and sputum (strain TXO6–TXO10) of humans were of similar morphology. The cells were ellipsoidal or ovoid in shape measuring 6·5–8·0 μm × 5·0–8·0 μm. Non-dividing organisms possessed 4 subequal anterior flagella and 1 recurrent flagellum associated with the undulating membrane and continuing as a free posterior flagellum beyond its end. A well-developed undulating membrane supported by a relatively stout costa extended for the entire length of the body. The capitulum of the axostyle was spatulate and relatively wide, with the axostylar trunk of uniform diameter along its length protruding posteriorly in a short cone-shaped projection (Fig. 1A). The ellipsoidal nucleus measured 1·5–1·8 μm × 1·0–1·5 μm. The parabasal apparatus was discoid (Fig. 1B), typically with a central granule. From this description it is apparent that the Estonian strains do not belong to the *T. tenax* species. Their morphological characters conform to the genus *Tetratrichomonas*, morphologically similar to the avian species *Tetratrichomonas gallinarum* (Martin and Robertson, 1911), but significantly differ from those of the genus *Trichomonas* (Table 2). The gross morphology of the Estonian tetratrichomonads and *T. tenax* is documented in Fig. 1.

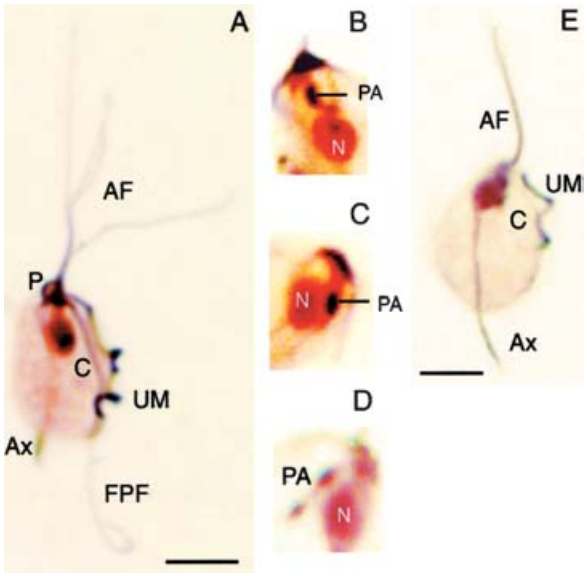

Fig. 1. Morphological comparison of *Tetratrichomonas* sp. and *Trichomonas tenax*. (A) Cell of *Tetratrichomonas* sp. – oral strain TXO7. (B–C) Detail of parabasal apparatus of *Tetratrichomonas* sp. – bronchial strain TXB3. (D) Detail of parabasal apparatus of *Trichomonas tenax* Hs-4:NIH. (E) Cell of *Trichomonas tenax* Hs-4:NIH. AF, anterior flagella; Ax, axostyl; C, costa; FPF, free posterior flagellum; N, nucleus; P, pelta; PA, parabasal apparatus; UM, undulating membrane.

Comparative genetic analysis of bronchial and oral strains by RAPD

As the study of the cell morphology did not reveal any differences between bronchial and oral isolates, analysis of PCR-amplified anonymous DNA fragments was chosen as a more sensitive method. The RAPD analysis separated Estonian strains into 2 distinct groups, each providing characteristic patterns of DNA fragments (Fig. 2). The 'bronchial' group included all 5 bronchial isolates (TXB1–TXB5) as well as 2 isolates collected from oral cavities (TXO9–TXO10). The 'oral' group consisted of 3 oral isolates (TXO6–TXO8). Distinct species-specific patterns were observed for *Trichomonas vaginalis*, *Trichomonas gallinae*, *Pentatrichomonas hominis* and *Tritrichomonas foetus* (Fig. 2). The data from RAPD analyses using all 9 primers, which provided altogether 838 DNA fragments were used for construction of a dendrogram (Fig. 3). The 'bronchial' and 'oral' groups were separated into distinct clades, each with high bootstrap support (100%). The dendrogram also confirmed separation of the Estonian isolates from all other trichomonad species involved in the RAPD analysis including 2 representatives of the genus *Trichomonas* (*T. vaginalis* and *T. gallinae*).

Analysis of ITS1-5.8S rRNA-ITS2 regions

To establish the taxonomic position of the Estonian strains within the Trichomonadinae subfamily,

Table 2. Main morphological characters of the genera *Trichomonas* and *Tetratrichomonas*

Character	Trichomonas	Tetratrichomonas
Anterior flagella	Four	Four
Undulating membrane	Shorter than the body	Extending for a full length of the body
Recurrent flagellum	Associated with the undulating membrane, does not continue behind its end	Associated with the undulating membrane, continues behind its end as a free flagellum
Parabasal body	Rod- or V-shaped	Typically discoid, often with central granule

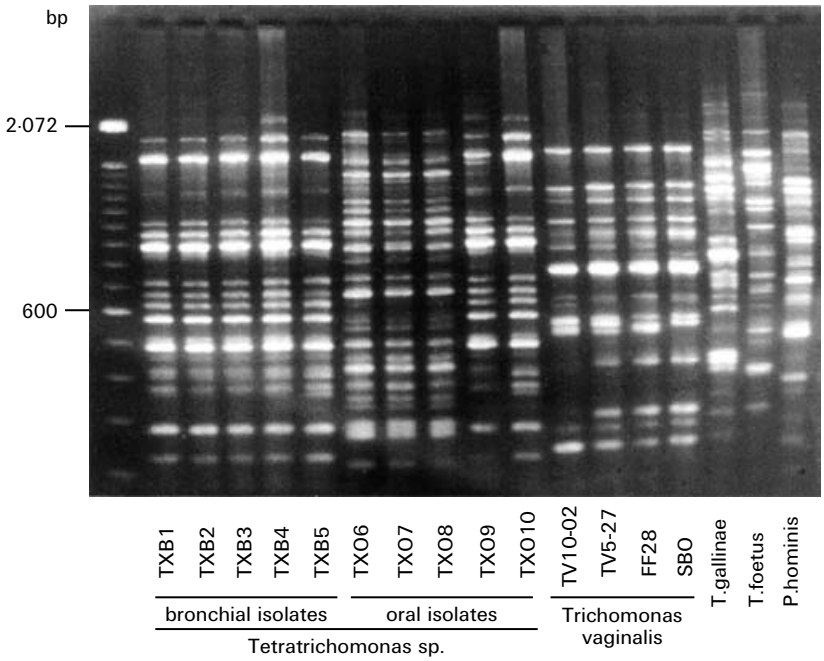

Fig. 2. Results of RAPD amplification with primer UBC 174. Strains TXB1–TXB5 and TXO 9–TXO10 represent the 'bronchial' group, strains TXO6–TXO8 the 'oral' group. For specification of other used strains see Table 1.

sequences of 5.8S rRNA genes and the flanking internal transcribed ITS1 and ITS2 regions were analysed. Complete sequences of ITS1-5.8S rRNA-ITS2 regions of 328 and 330 bp were determined for 3 strains of the 'bronchial' (TXB3, TXB5, TXO9) and the 'oral' (TXO6, TXO7, TXO8) groups, respectively. As the morphological data indicated, these strains belong to the genus *Tetratrichomona*s. We also determined nucleotide sequences of 3 strains of *Tetratrichomonas gallinarum* isolated from duck (*Anas platyrhynchos*), turkey (*Meleagris gallopavo*), and fowl (*Gallus gallus*), *Tetratrichomonas limacis* from garden slug (*Limax maximus*) and *Tetratrichomonas prowazeki* from slow worm (*Anguis fragilis*), the type species of the genus. The set of data further included *Tetratrichomonas* sp. isolated from duck by Crespo *et al*. (2001) and 3 tetratrichomonad strains isolated from the bovine preputial cavity (Walker *et al*. 2003). In addition to tetratrichomonads, the analysis included 4 species of the genus *Trichomonas* including oral species from a human and a dog, *Trichomonas tenax* and *Trichomonas canistomae*, respectively (sequence

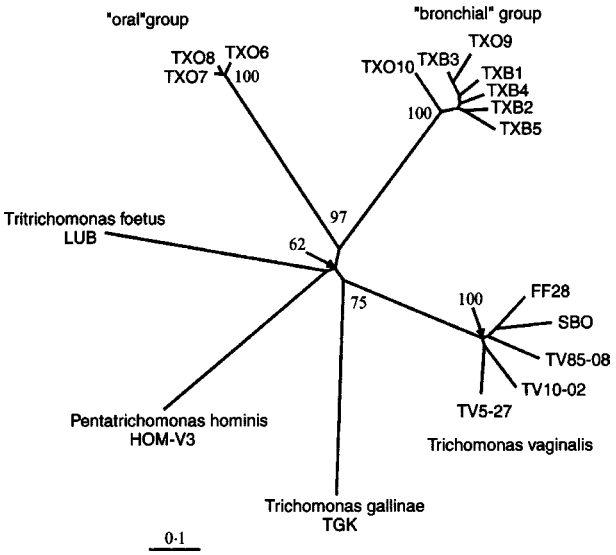

Fig. 3. Tree based on RAPD PCR amplification. 'Oral' and 'bronchial' groups of *Tetratrichomonas* sp. form separate branches supported with high bootstraps, distinct from *Trichomonas vaginalis* group and other included strains.

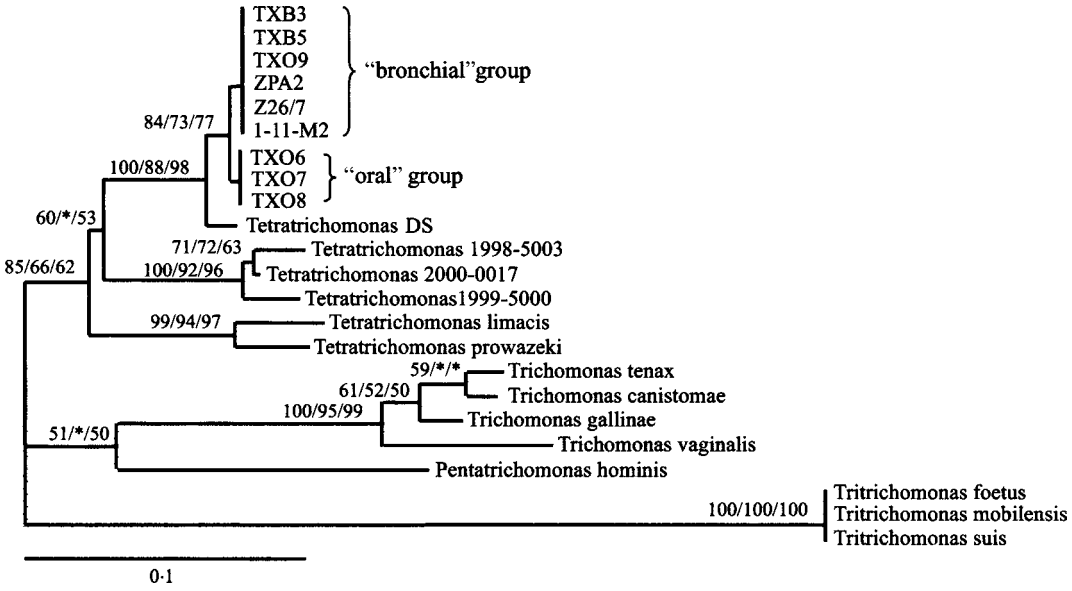

Fig. 4. Trichomonad tree based on comparison of the ITS1-5.8S rRNA-ITS2 region sequences. The tree was constructed using the neighbor-joining/Maximum Likelihood/Maximum Parsimony methods. Asterisks designate nodes with bootstrap values below 50%.

determined in this study), and *Pentatrichomonas hominis*. *Tritrichomonas* species were used as the outgroup. The Estonian strains formed 2 closely related subgroups of tetratrichomonad clade corresponding to the 'oral' and 'bronchial' groups revealed by RAPD analysis (Fig. 4). In addition to strains TXB3, TXB5 and TXO9, the 'bronchial' subgroup also included 3 strains of *Tetratrichomonas gallinarum* (ZPA2, Z26/7, 1-11-M2). The complete nucleotide sequences of all members of this subgroup were 100% identical. Also, no sequence differences were observed among 3 strains of the 'oral' subgroup, which differed from the bronchial group at 9 positions: 2 deletions and 3 transversions (A-C) in the ITS1 region, 2 transitions (C-T and A-G) in the 5.8S rRNA gene, and 1 transition (A-G) and 1 transversion (G-T) in the ITS 2 region (Fig. 5). Interestingly, *Tetratrichomonas* sp. from a duck was distinct from 3 strains of *Tetratrichomonas gallinarum* and it was placed as an outgroup to 'oral' and 'bronchial' subgroups. Tetratrichomonads from the bovine preputial cavity formed a separate subgroup that appeared at the sister position of the 'bronchial' and 'oral' subgroups. *T. limacis* and *T. prowazeki* formed the most divergent group of all tetratrichomonads containing 2 and 3 insertions, respectively, which were not present in other trichomonad species. As expected, *Trichomonas tenax* was placed within a separate *Trichomonas* clade with *Trichomonas canistomae* as a sister species. These results confirmed that all Estonian strains belong to the genus *Tetratrichomonas* and indicated that these organisms are closely related to, if not identical with the intestinal parasite of birds, *Tetratrichomonas gallinarum*.

Experimental infections

To examine the infectivity of the human tetratrichomonad strains for avian hosts, the bronchial TXB5 and oral TXO6 strains were inoculated intracloacally into turkey poults. *Tetratrichomonas gallinarum* strain 1-11-M2 of turkey origin was used as a positive control. The animals were sacrificed 10 days after inoculation and examined at autopsy. All poults inoculated with the control *T. gallinarum* strain became infected. They showed watery caecal content containing numerous motile trichomonads. In contrast, both TXB5 and TXO6 strains failed to establish an infection. No trichomonads were observed in a formed caecal contents at microscopic examination, and attempts to re-isolate trichomonads in TYM medium were negative from all birds of these latter groups.

DISCUSSION

Genetic analysis as well as morphological examination of axenic trichomonad strains isolated in Estonia from patients with chronic respiratory diseases revealed that these organisms represent novel trichomonad species. All examined Estonian strains showed typical morphology of the genus *Tetratrichomonas*. They significantly differ from *T. tenax*, and they were unequivocally separated by RAPD analysis from *Trichomonas vaginalis* and *Pentatrichomonas hominis* as well as from 2 pathogenic species from animals *Tritrichomonas foetus* and *Trichomonas gallinae*. In a dendrogram based on sequence analysis of the ITS1-5.8S rRNA-ITS2 region, these strains clustered with other members

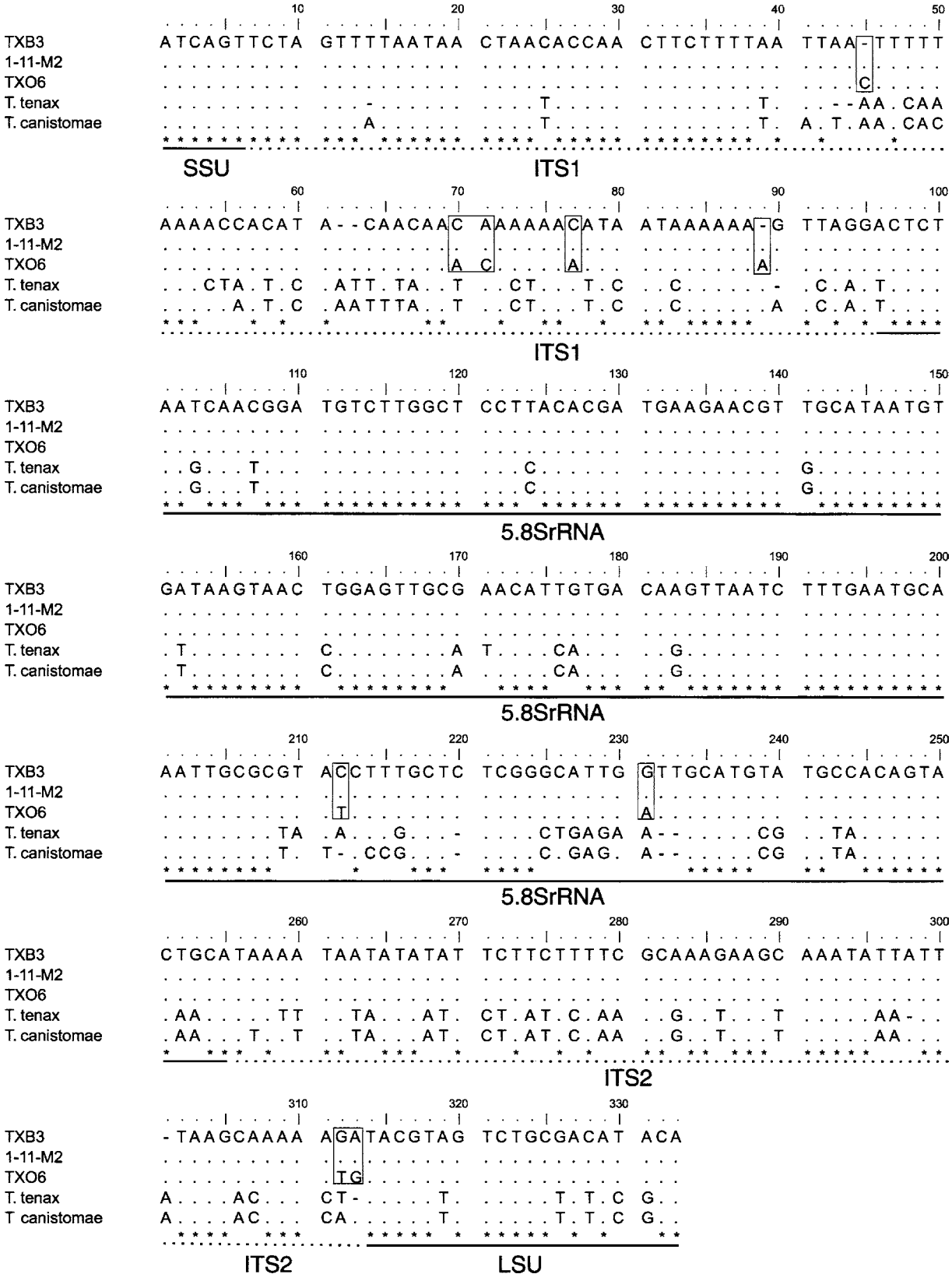

Fig. 5. Alignment of sequences of the ITS1-5.8SrRNA-ITS2 region of *Tetratrichomonas* sp. strain TXB3 (bronchial group) and TXO6 (oral group), *Tetratrichomonas gallinarum* strain 1-11-M2 and *T. canistomae* (strain BRIXI). Differences between oral and bronchial isolates are marked with boxes.

of the genus *Tetratrichomonas* to form a monophyletic clade of the genus. Sequences of 3 strains of the 'bronchial' group and 3 strains of the 'oral' group showed 100% and 95% identity, respectively, with the 3 strains of *Tetratrichomonas gallinarum* (ZPA2, Z26/7, 1-11-M2) isolated from duck, fowl

and turkey. The members of the genus *Trichomonas* including *T. vaginalis* and oral parasites of humans (*T. tenax*) and dog (*T. canistomae*) formed a distinct, statistically well-supported clade.

Why were only tetratrichomonads found among the archival axenic strains isolated in Tallin? According to Teras *et al.* (1980), numerous xenic trichomonad isolates were obtained during the Tallin study that was aimed at revealing the aetiological role of *Trichomonas tenax* in human lung pathologies. All were assumed to be *T. tenax* and it is likely that the true *T. tenax* isolates were present among them. Axenization of some of these cultures was later attempted with variable success and axenic cultures only were further maintained for use in laboratory experiments. The authors employed their original technique of axenization using a complex TT-1 medium with antibiotics, gradual transition from biphasic to monophasic culture and U-tube migration (Teras *et al.* 1970). It is generally known that unlike some other trichomonad species, *T. tenax* is rather difficult to axenize. A critical step in *T. tenax* axenization is pre-adaptation of the trichomonads to bacteria-free conditions in monoxenic culture with a kinetoplastid flagellate such as *Trypanosoma cruzi* (Diamond, 1962) or *Crithidia* sp. (Feki and Molet, 1982). It can be assumed that the method employed by the Estonian workers, that did not involve the monoxenic cultivation step, might exert a negative selection pressure against *T. tenax*, while permitting axenic growth of tetratrichomonads. It should be noted in this context that attempts to establish axenic cultures directly from the bacterial xenic stage by us as well as by others were unsuccessful (Diamond, 1962; Ribaux, 1979). Repeated attempts in the Prague laboratory to axenize local isolates of *T. tenax* by adhering strictly to the protocol provided by Teras were also unsuccessful (Urbánková and Kulda, unpublished observations).

Differences between the oral and bronchial strains

Roigas *et al.* (1980) and Kazakova *et al.* (1985) reported differences in antigenic properties between their isolates from the oral cavity and bronchi of humans. Although we found that all the examined Estonian strains are morphologically indistinguishable, our DNA analyses confirmed the presence of 2 subsets of organisms assigned as the 'oral' and the 'bronchial' groups. The RAPD technique has previously been shown to provide species and/or strain-specific 'fingerprints' of trichomonads (Vanacova *et al.* 1997; Tachezy *et al.* 2002). This technique separated the 'oral' group (consisting of oral isolates only) from the 'bronchial' group that included all bronchial isolates and 2 oral isolates. This separation was confirmed by sequencing of ITS1-5.8S rRNA-ITS2 regions.

Relationships of the Estonian strains to Tetratrichomonas gallinarum

Our morphological observations and genetic analyses have shown a very close relationship of the human tetratrichomonad strains with a common avian species *T. gallinarum*. There is strong support for the placement of these strains into subgroups of *T. gallinarum* isolates from domesticated galliform or anseriform birds as confirmed by more extensive genetic analyses involving additional *Tetratrichomonas* species and strains (Cepicka *et al.* 2005).

To test whether these birds might be natural hosts of tetratrichomonads isolated from humans, transmission of the bronchial strain TXB5 and the oral strain TXO6 to turkey poults was attempted by intracloacal inoculation. Susceptibility of turkey poults to experimental infection by this route has been reported previously for various axenic strains of *T. gallinarum* isolated from a ducks, geese and hens (Kulda *et al.* 1974). Failure of our attempts to transmit tetratrichomonads of human origin, both bronchial (TXB5) and oral (TXO6), to turkey poults, while the turkey *T. gallinarum* strain (1-11-M2) infected all tested animals, indicates biological separation of the human-host-adapted *T. gallinarum*-like trichomonads. Accordingly a current study on *T. gallinarum*-like trichomonads (Cepicka *et al.* 2005) showed that human strains pertinent to a subgroup that includes also trichomonads from galliform and anseriform birds, form an independent, host-specific branch within this subgroup. It cannot be completely ruled out that the failure to infect avian hosts by the human tetratrichomonads resulted from a loss of their infectivity due to prolonged *in vitro* cultivation. Such an explanation, however, seems unlikely in light of findings on virulence attenuation of related bird pathogens *Trichomonas gallinae* and *Histomonas meleagridis* that maintained their infectivity for avian hosts after prolonged *in vitro* cultivation (Stabler, Honigberg and King, 1964; Dwyer and Honigberg, 1970, 1972).

The question of pathogenicity of the Estonian strains

All Estonian strains examined in this study were isolated from patients with chronic pulmonary disorders. According to Estonian workers, patients with pulmonary infection by these parasites presented specific humoral response at the serological examination (Kazakova *et al.* 1979, 1980; Roigas *et al.* 1980) and reacted positively in intradermal test for delayed hypersensitivity with corpuscular antigen from axenic Estonian isolates but not with those from *Trichomonas vaginalis* or *Pentatrichomonas hominis* (Sardis *et al.* 1983). Intraperitoneal inoculation of the axenic isolates into laboratory mice induced self-healing fibrinous peritonitis with transient exudative

period and localized inflammatory foci that were eventually encapsulated (Sardis *et al*. 1982). No invasions of internal organs typical for infections with virulent strains of *T. vaginalis* or *T. gallinae* were observed. However, the possibility of virulence attenuation by prolonged *in vitro* cultivation should be considered. More recently, Bozner and Demes (1991*a*) characterized cell-associated as well as secreted proteolytic activities of the Estonian TXB1 strain (reported as *Trichomonas tenax*). The major proteolytic activity was ascribed to cysteine proteinases. These authors also showed activity of secreted proteinases against 4 native types of collagens (Bozner and Demes, 1991*b*), a property that may further support presumable pathogenic potential of the tetratrichomonad strains for humans. It is tempting to speculate that trichomonads isolated in Tallin that were identified in this study to be members of the *Tetratrichomonas* genus might be responsible for pulmonary pathologies ascribed to *T. tenax*. However, equivocal evidence on their primary pathogenicity is not yet available.

Conclusions

Results presented in this paper identify a novel trichomonad flagellate from lungs and the oral cavity of humans that has not previously been recognized as a human parasite. The organism belongs to the genus *Tetratrichomonas* being closely related to the avian species *Tetratrichomonas gallinarum*. We refrain, at present, from naming the species as the taxonomy of the *Tetratrichomonas* genus is unsettled. Current data indicate that *T. gallinarum* apparently is a species complex comprising several major groups, each with additional cryptic taxons (Cepicka *et al*. 2004). The human isolates clearly belong to the *T. gallinarum* complex. Further studies are required to clarify (i) whether the human infections are zoonotic and if so, which avian host may serve as a source of infection (ii) how frequent the human infections by tetratrichomonads are (iii) what their geographical distribution is, and (iv) and what is the pathogenic potential of these parasites.

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