Demonstration of Brachyspira aalborgi lineages 2 and 3 in human colonic biopsies with intestinal spirochaetosis by specific fluorescent in situ hybridization

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Sequences of known 16S rRNA genes, derived from sequence analysis of cloned 16S rDNA, were used to design a specific oligonucleotide probe targeting spirochaetes of Brachyspira aalborgi lineages 2 and 3. The probe was used with fluorescent in situ hybridization to study the involvement of these organisms in human intestinal spirochaetosis. Seventeen human colonic biopsies from Norway and Denmark with intestinal spirochaetosis caused by Brachyspira-like organisms different from the type strain of B. aalborgi (lineage 1) were examined. Application of the probe gave a positive signal in two Norwegian biopsies, whereas the 15 other biopsies were hybridization-negative. The positive reaction visualized the spirochaetes as a fluorescent, 3–5 μm-high fringe on the surface epithelium, extending into the crypts. The study verified the presence of B. aalborgi lineages 2 and 3 and identified the bacteria as an aetiological agent of human intestinal spirochaetosis.

Introduction

Human intestinal spirochaetosis (HIS) is a condition of the large intestinal mucosa microscopically characterized by colonization and extensive end-attachment of densely packed spirochaetes to the epithelial surface as a bluish fringe in haematoxylin and eosin-stained sections (Harland & Lee, 1967). To date, only the two anaerobic intestinal spirochaetes Brachyspira aalborgi (Hovind-Hougen et al., 1982) and Brachyspira pilosicoli (Trott et al., 1996) have been identified as aetiological agents of HIS (Mikosza et al., 1999; Trivett-Moore et al., 1998). In a recent study, application of in situ hybridization identified Brachyspira aalborgi in 55 % (21/38) of colorectal biopsies with histological evidence of HIS (Jensen et al., 2001). The spirochaetes in the remaining 45 % of the biopsies hybridized only with a Brachyspira genus probe, indicating that they belonged to the genus Brachyspira but differed from the known type strains of B. pilosicoli (ATCC 51139T) and B. aalborgi (ATCC 43994T) (Jensen et al., 2001). Based on phylogenetic analysis of cloned 16S rRNA genes, human intestinal spirochaetes (Brachyspira) can now be divided into three distinct lineages and a heterogeneous group indistinguishable from B. pilosicoli (Pettersson et al., 2000). The first lineage comprises the type strain of B. aalborgi and the only other isolates of B. aalborgi cultured so far (Hovind-Hougen et al., 1982; Kraaz et al., 2000), whereas the as yet uncultured spirochaetes in lineages 2 and 3 harbour clones in which the 16S rDNA sequences differ from those of lineage 1.

Alignment of the 16S rRNA sequences of B. aalborgi lineages 2 and 3 with the sequence of an 18-bp oligonucleotide probe (S-S-B.aalborgi-0183-a-A; Jensen et al., 2001) reveals four mismatches; thus, the spirochaetes will not be detected by the probe for B. aalborgi type strain/lineage 1.

The involvement and importance of the new lineages of B. aalborgi in HIS has yet to be determined, as Pettersson et al. (2000) only detected DNA from organisms in colonic mucosa samples from two individuals without clinical symptoms.

The aim of this survey was to design an oligonucleotide probe that specifically targets lineages 2 and 3 of B. aalborgi to determine by in situ hybridization the involvement of these organisms in HIS.

Methods

Based on the cloned 16S rRNA genes published by Pettersson et al. (2000), a specific oligonucleotide probe targeting lineages 2 and 3 of B. aalborgi was selected by using the function Probe Design in the software ARB (Ludwig et al., 2002).
The probe was purchased from MWG-Biotech AG, synthesized by standard phosphoramidite chemistry, 5’-labelled with an aminoethyl linker and conjugated to FITC. The probe was applied to 17 formalin-fixed, paraffin-embedded colorectal biopsies with histologic evidence of HIS (presence of the characteristic, 3–6 μm thick, bluish fringe in haematoxylin and eosin-stained sections). The biopsies had previously been found to be hybridization-negative for *B. aalborgi* ATCC 43994T and *B. pilosicoli* ATCC 51139T by in situ hybridization, but positive for *Brachyspira* spp. as described by Jensen et al. (2001). Using the nomenclature of the original work, biopsies 15–25, 39 and 41–45 were examined.

Six of the biopsies were from Norway and the other 11 were from Danish patients. Hybridization was carried out as described previously (Jensen et al., 2001). Prior to hybridization, the sections were deparaffinized in xylene and transferred to 96 % ethanol for 10 min. Hybridization was carried out at 37°C with 20 μl hybridization buffer (100 mM Tris/HCl, pH 7.2, 0.9 M NaCl, 0.1 % SDS) and 100 ng probe for 16 h in a moisture chamber. The samples were then washed in 100 ml prewarmed (37°C) hybridization buffer for 15 min and subsequently in 100 ml prewarmed (37°C) washing solution (100 mM Tris/HCl, pH 7.2, 0.9 M NaCl) for 15 min. The samples were rinsed in water, air-dried and mounted in Vectashield (Vector Laboratories) for fluorescence microscopy.

An Axioplan 2 epifluorescence microscope (Carl Zeiss) equipped for epifluorescence with a 75-W Xenon lamp and a double filter set (XF53; Omega Optical) was used for simultaneous detection of red and green fluorescence. Images were taken with a CoolSnap colour CCD camera (Media Cybernetics).

**Results and Discussion**

A specific oligonucleotide probe (5’-CTACACTTATGTGTC AAG-3’) was designed for clusters 2 and 3 of the *B. aalborgi* lineage and given the systematic name S-S-B.x-0183-a-a-18 (for simplicity, usually referred to as Bx183). It has four mismatches with the previous published probe for *B. aalborgi* (S-S-B.aalborgi-0183-a-A, with the sequence 5’-CTACG CTTTAGCTCAAG-3’). The latter probe targets cluster 1 of the *B. aalborgi* lineage only.

Hybridization with probe Bx183 gave a positive signal in two biopsies (39 and 44), whereas the 15 other biopsies were hybridization-negative. The positive reaction visualized the spirochaetes as a green fringe on the surface epithelium, as illustrated in Fig. 1. The fringe was also sometimes seen extending a short distance into the crypts. The fringe, 3–5 μm high, appeared uniform; even at high magnification, it was not possible to distinguish single spirochaetes. The two positive biopsies were Norwegian and came from a 76-year-old woman and a 36-year-old man with adenocarcinoma and diarrhoea, respectively. Histopathologically, the spirochaetal fringe observed in the two biopsies was similar to that found in *B. aalborgi*-positive biopsies and to that in biopsies positive for the genus *Brachyspira* only (Jensen et al., 2001).

As the fringe of spirochaetes in the two biopsies showed only a relatively weak signal, similar to that observed with the *Brachyspira* genus probe (Jensen et al., 2001), observation of individual cells not associated with the surface attachment was not expected.

The analysis by Pettersson et al. (2000) of cloned 16S rRNA genes from intestinal spirochaetes allowed us to demonstrate the presence of phylogenetic *B. aalborgi*-like organisms in the distal colonic mucosa from two Swedish adult individuals without clinical symptoms or history of HIS. Together with the work by Mikosza et al. (2004) describing partial sequence analysis of the 16S rRNA gene of spirochaetes in biopsies with HIS, this study verifies the existence of the new lineages of *B. aalborgi*. In addition, this study is the first to identify *B. aalborgi* lineages 2 and 3 as an aetiologic agent of HIS.

With the demonstration of *B. aalborgi* lineages 2 and 3 in these two cases, species identification of the spirochaetes in the remaining 15 biopsies from Jensen et al. (2001) awaits further phylogenetic analysis. At present, there is relatively little knowledge regarding strain diversity of *Brachyspira* species, but, in this issue, Mikosza et al. (2004) describe a fourth lineage of *B. aalborgi* obtained from faecal samples of non-human primates.

Further studies to identify other as yet uncharacterized *Brachyspira* organisms associated with HIS may include PCR amplification of spirochaetal DNA from microdissected biopsies.

**References**


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