Aus dem Institut für Mikrobiologie und Hygiene
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DISSERTATION

In Situ Detection, Molecular Epidemiology, and Improvement of Molecular Tools for the Understanding and Diagnosis of Infections Caused by Spirochetes

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1. Summary

1.1. Abstract in German

Pathogene Spirochäten sind Erreger von weltweit, auftretenden Infektionskrankheiten wie der humanen intestinalen Spirochätose (HIS), Syphilis, Lyme-Borreliose und Leptospirose. Dabei führen die einschränkten Isolierungs- und Kultivierungsmöglichkeiten dieser anspruchsvollen Mikroorganismen zu Unter- und Fehldiagnosen der Infektionen. Fluoreszenz-*in-situ*-Hybridisierung (FISH), eine Technik zur gleichzeitigen Visualisierung und Identifizierung von Mikroorganismen in Biopsieproben, ist ein akkurates Instrument für die Diagnose und das Verständnis der Pathogenität verschiedener Infektionen. In Kombination mit anderen molekularen Methoden, wurde FISH zur Diagnose der HIS und die Identifizierung von *Treponema pallidum* subsp. *pallidum* - dem Erreger der Syphilis - in klinischen Fällen eingesetzt.

Brachyspira spp., der Infektionserreger von HIS, ist eine langsam wachsende und anspruchsvolle anaerobe Spirochete, die die menschliche Kolonmukosa kolonisiert. Die aktuelle Routine zur klinischen Diagnose von HIS beinhalten die Histopathologie-Analyse ohne molekulare Identifizierung der Erreger. Die Genus-spezifische FISH wurde in Kombination mit *16S rRNA*-Gen PCR und Sequenzierung auf 149 Darmbiopsien von 91 Patienten angewendet, welche zuvor mit HIS durch Histopathologie diagnostiziert worden waren. Die Bakteriengattung *Brachyspira* wurde bei 77 Patienten identifiziert. Von diesen konnten 53 den *B. aalborgi*, 23 *B. pilosicoli*, einer gemischten Infektion und 14 FISH- und PCR-negativen Fällen zugeordnet werden. Vier der 14 negativen Fälle zeigten eine Kolonisation des Darmepithels nur durch Stäbchen- und Kokken, was auf eine Fehldiagnose durch Histopathologie hindeutet.

Syphilis ist eine humane Multisystem-Erkrankung mit atypischen klinischen Manifestationen und komplexer Diagnose. Der Erreger der Syphilis ist das Bakterium *Treponema pallidum* und ist noch nicht erfolgreich in vitro kultiviert worden. Der Erreger verbreitet sich durch den menschlichen Körper über das Kreislaufsystem. Obwohl die Serologie derzeit zur Identifizierung verschiedener Stadien der Infektion verwendet wird, können die Ergebnisse aufgrund einer Überschneidung der Zeichen und Symptome schwer zu interpretieren sein. Ein spezies-spezifischer zur 16S-rRNA-Gen-Markierung entworfener FISH-Experiment-Sonden wurde verwendet, um die Anwesenheit von *T. pallidum* in vier verschiedenen Gewerbebiopsien zu zeigen: ein Leisten-Lymphknotengewebe eines HIV-Patienten, ein präputiales Gewebe eines Patienten nach Phimose OP, eine Mundschleimhautbiopsie und eine Gewebebiopsie aus Tonsillen.

Zusätzlich wurden rekombinante *Borrelia burgdorferi* Bakterienstämme, die Erreger der Lyme-Borreliose, verwendet, um die Bindungsrolle des Kandidaten-Adhäsionsproteins Tp0751 (Pallilysin) aus *T.pallidum* als fördernder Faktor zur bakteriellen Verbreitung in Tiermodellen zu verstehen. Vier verschiedene Bakterienstämme von *B. burgdorferi* wurden verwendet, um die Funktion von Tp0751 in in vitro und in vivo Experimenten zu beurteilen. Mit der Verwendung des Mikroskopischen Videotracking-System wurde die Bindungsinteraktion von Spirochäten unter verschiedenen Bedingungen quantifiziert. Die Ergebnisse zeigten, dass Tp0751 ein Adhäsin ist, das die bakterien-vaskuläre Wechselwirkungen wiederherstellen und eine Rolle bei der Bakterienverbreitung spielen könnte.

Diese Dissertation untersucht die Vorteile mikroskopischer Techniken bei der Untersuchung von Spirochäten-Infektionen. FISH zeigt die Invasivität und räumliche Verteilung von *T. pallidum* in Syphilis und unterstützt bei der Diagnose von HIS. Intravitale Mikroskopie hilft zudem bei der Identifikation von Molekülen und Virulenz Faktoren, die eine Rolle bei der Verbreitung der Bakterien in Syphilis- und Lyme-Borreliose spielen.

1.2. Abstract in English

Pathogenic spirochetes are the causative agents of some re-emerging diseases with global significance such as human intestinal spirochetosis (HIS), syphilis, Lyme disease and leptospirosis. Limitations in isolation and cultivation of these microorganisms lead to under- and misdiagnosis of bacterial infections. Fluorescence *in situ* hybridization (FISH), a technique designed to simultaneously visualize and identify microorganisms in biopsy samples, is an accurate microscopy tool for the diagnosis and understanding of pathogenicity in several infections. FISH, in combination with other molecular methods, was applied for the diagnosis of HIS and the identification of *Treponema pallidum*, the causative agent of syphilis.

Brachyspira spp., the infectious agent of HIS, is a slow growing and fastidious anaerobe spirochete that colonizes the human colonic mucosa. Current routine clinical diagnosis of HIS involve histopathology analysis without molecular identification of the causative agent. Genus-specific FISH, in combination with *16S rRNA* gene PCR and sequencing were applied to 149 intestinal biopsies from 91 patients, previously diagnosed with HIS by histopathology. The genus *Brachyspira* was identified in 77 patients, 53 of which cases could be assigned to the *B. aalborgi* lineage, 23 to *B. pilosicoli*, one mixed infection and 14 FISH and PCR negative cases. Four out of the 14 negative cases showed colonization of the intestinal epithelium by rods and cocci only, suggesting a misdiagnosis by histopathology.

Syphilis is a multi-systemic disease characterized by its atypical clinical manifestations and complex diagnosis. Its causative agent, *Treponema pallidum,* disseminates through the body via the circulatory system and has not yet been successfully cultured *in vitro.* Although serology is currently used for the identification of different stages of infection, the results may be difficult to interpret due to an overlap of the signs and symptoms. A species-specific FISH probe assay, designed to target the *16S rRNA* gene, was used to show the presence of *T. pallidum* in four different tissue samples: lymph node tissue of an HIV patient, preputial tissue, oral mucosa, and tonsils.

Additionally, recombinant strains of *Borrelia burgdorferi*, the causative agent of Lyme disease, were used to understand the binding role of candidate adhesion protein Tp0751 (pallilysin) from *T. pallidum* as a factor promoting bacterial dissemination in animal models. Four different strains of *B. burgdorferi*, were used to assess the function of Tp0751 in *in vitro* and *in vivo* experiments. With the use of video tracking microscopy, the binding interaction of spirochetes was quantified in different conditions and showed that Tp0751 is an adhesin capable of restoring bacteria-vascular interaction that may play a role in bacterial dissemination.

This dissertation explores the benefits of microscopy techniques for the understanding of infections caused by spirochetes. FISH facilitates the accurate diagnosis of HIS and provides insights about the invasiveness and spatial distribution of *T. pallidum* in syphilis. Lastly, intravital and video tracking microscopy helps in the identification of molecules linked to virulence factors related to motility and bacterial dissemination in syphilis and Lyme disease.

1.3. Introduction

This dissertation comprises three studies in the field of clinical microbiology related to the diagnosis and understanding of the molecular pathogenicity of diseases caused by spirochetes using microscopy techniques [1-3].

Fluorescence *in situ* hybridization (FISH) is a molecular technique that allows simultaneous visualization and identification of microorganisms in situ[4]. It has so far been applied to a broad range of clinical specimens yielding valuable information about the presence of pathogenic, fastidious and yet uncultivable bacteria in human biopsy samples [1, 2, 4-6]. This document summarizes the application of FISH, by the German National Reference Laboratory for *Treponema* Identification, for the diagnosis of human intestinal spirochetosis (HIS) in a period of six years [1], and the identification of *Treponema pallidum* subsp. *pallidum* in syphilis cases where serology was inconclusive [2]. Lastly, this document´s findings shed light into the role of *T. pallidum* surface protein Tp0751 (pallilysin), in the restoration of bacterial-vascular interaction of recombinant *B. burgdorferi*, pointing to the importance of adhesion proteins (adhesins) in bacterial dissemination. Flow chamber assays were conducted using static and controlled fluid shear stress conditions to observe the interaction between recombinant *B. burgdorferi* strains and human umbilical vein endothelial cells (HUVEC). Complementary *in vivo* experiments were conducted using the same recombinant strains in post capillary venules (PCVs) in a mouse model where visualization was performed using intravital microscopy (IVM) [3].

The etiological agent of HIS is an anaerobe spirochete called *Brachyspira* spp., It colonizes the luminal surface of the large intestine [7, 8] causing abdominal pain, long-lasting diarrhea and rectal bleeding [7, 9, 10], where previous studies have reported symptoms improvement after antibiotic treatment [8, 11]. HIS has a worldwide distribution and a prevalence ranging from 2.5 to 62.5% in risk groups, such as men who have sex with men (MSM) and HIV-positive individuals [12-17]. Although epidemiological analysis and clinical relevance of intestinal spirochetosis in pigs and poultry are well documented [18, 19], only few studies have been performed on human biopsies. So far, two species of *Brachyspira* have been identified in clinical human samples [20]; *B. aalborgi* [21] and *B. pilosicoli* [22], both separately [15, 23, 24], and in mixed infections [23, 25]. Moreover, sequence analysis of clinical isolates from HIS patients revealed putative clusters of strains specific to humans for both *B. pilosicoli* [6] and *B. aalborgi* [23, 26, 27]. HIS is usually diagnosed with histopathology by visualizing a “false brush border” resembling a 4-7 µm thick basophilic fringe, comprised of end-on attached *Brachyspira* spp. to the luminal border of the enteric epithelia. In contrast to other unspecific methods used in histopathology, such as hematoxylin and eosin (HE), Warthin-Silver-stain and periodic acid-Schiff (PAS) [7, 28], the oligonucleotide FISH probes that target *Brachyspira* spp. 16S or 23S rRNA have previously provided genus-specific results when applied to porcine and human tissues [6, 29-32], as well as for the identification of other spirochetes in diverse clinical samples [2, 33-36].

Syphilis is a worldwide re-emerging sexually transmitted disease characterized for its atypical signs and symptoms. It is caused by the spirochete *T. pallidum*, which has not yet been successfully cultured *in vitro*. Every year 11 million are reported worldwide, which poses a great public health concern [37] as well as it contributes to an increase HIV transmission and acquisition in humans with an active *T. pallidum* infection [38]. Although *T. pallidum* can be directly identified in clinical specimens with dark field microscopy (DFM) and direct fluorescence assay (DFA) [39], additional histopathology techniques such as Warthin–Starry and immunohistochemistry methods [40], demand exhaustive analysis to avoid false positives due to cross reactivity with other microorganisms [41]. Hence, clinical diagnosis, determination of disease stage and treatment efficacy of syphilis rely on a combination of serological tests and assays such as *T. pallidum* particle agglutination/haemagglutination (TPPA/TPHA), fluorescent treponemal antibody absorption (FTA-ABS), and Venereal Disease Research Laboratories (VDRL) [42-46]. Unfortunately, samples from immunosuppressed patients tend to yield inconclusive serology. FISH, previously used for the identification of other spirochetes such as *Borrelia* spp, and oral treponemes [33-36, 47, 48] could provide insight regarding the localization, dissemination and tissue invasiveness of *T. pallidum* in biopsy samples from cases with complex clinical manifestations.

The inability to cultivate *T. pallidum* has hampered the application of experimental tools, such as genetic engineering, in the characterization of pathogenicity-related molecules and identification of virulence factors involved in disease progression. Dissemination and microbial adhesion to endothelial extracellular matrix (ECM) are suggested to be mediated by specialized bacterial adhesins such as BBK32 in *B. burgdorferi* [49] and Tp0751 in *T. pallidum* [50-52]. These proteins enable circulating pathogens to overcome blood flow forces and bind to components of the inner surface of blood vessels hence facilitating transmigration and tissue invasion [50-52]. On these grounds, a recent study showed how adhesins Tp0751 and Tp0435 established host cell adhesion in recombinant non-adherent *Treponema phagedenis* and *B. burgdorferi* respectively [53, 54]. The invasive mechanisms used by *T. pallidum* to spread through the body, using the cardiovascular system, are still poorly understood [55] pointing to the importance of characterizing proteins involved in adhesion to the host ECM components is relevant for the development of strategies for vaccine development.

The three studies presented here [1-3] highlight the relevance of current molecular and microscopy methods in the diagnosis and understanding of pathogenic mechanisms involved in under- and miss-diagnosed treponemal diseases.

1.4. Objectives

1.4.1. General

To apply FISH as a combination and confirmation molecular diagnostics tool in the clinical diagnosis of conditions such as HIS and Syphilis. To determine the *Brachyspira*-species involved in HIS. To apply IVM and video tracking microscopy in the analysis of the role of adhesin candidate protein Tp0751 in bacterial dissemination.

1.4.2. Diagnosis of HIS

To report six years of HIS diagnostics findings by the German National Reference Laboratory for *Treponema* Identification. Also, to provide insights about the use of FISH in combination with PCR and 16S rRNA gene sequencing in the validation of histopathology findings for a better understanding of phylogeny and pathogenicity in human intestinal spirochetosis.

1.4.3. Diagnosis of syphilis

To investigate the localization, invasiveness and abundance of the causative agent of syphilis with the use of FISH for the identification and visualization of *T. pallidum* within tissues in clinical cases.

1.4.4. Insights about dissemination of *T. pallidum* in *in vitro* and *in vivo* models

To investigate whether *T. pallidum* adhesion protein Tp0751 has a bacterial-vascular interaction role in bacterial dissemination using microscopy *in vivo* and *in vitro* models.

1.5. Methodology

1.5.1. Common methods used for the diagnosis of human biopsy samples

1.5.1.1. Sample preparation and FISH

Analysis of clinical samples including preparation, mounting of the slides, hybridization with the respective oligonucleotide probe and microscopy were performed according to previously described methods [1, 2, 6]. Paraffin embedded samples were deparaffinated and stored in FISH fixation buffer solution as published [1, 6]. Sample embedding and sectioning was performed using cold polymerizing resin methacrylate (Technovit 8100; Heraeus Kulzer) according to the manufacturer's instructions [33]. FISH experiments were carried out using genus- or species-specific 5' end-labeled probes according to the study [1, 2], in addition to the pan-bacterial probe EUB338 (5 -GCTGCCTCCCGTAGGAGT-3) [56], and the "nonsense probe" NON338 (5'-ACTCCTACGGGAGGCAGC-3') which is the antisense probe of EUB338, used to exclude unspecific probe binding [57]. Digital images were obtained using an epifluorescence microscope Axioplan 2 (Carl Zeiss,) using an AxioCam MRm, employing AxioVision 4.6 software.

1.5.1.2. DNA extraction and PCR sequencing

A DNA extraction kit (Respiratory Specimen Preparation Kit, Amplicor, Roche) was used on 10-15 consecutive sections of the methacrylate embedded biopsies following the manufacturer's instructions. The details of the PCR specific programs and primers are describer elsewhere [1, 2]. The PCR products were sequenced by LGC Genomics and compared to previously published data in the EMBL and GenBank databases using the FASTA and BLAST tools (HUSAR 4.1 - Deutsches Krebsforschungszentrum).

1.5.2. Methods used in the analysis of human intestinal spirochetosis samples

1.5.2.1. Patient and sample characteristics

149 paraffin-embedded or native intestinal biopsies from 91 patients histologically diagnosed with HIS were investigated. Samples were sent from several institutes in Europe (mostly Klinikum Bayreuth GmbH, Germany) for confirmation of the diagnosis with FISH and PCR analysis. Intestinal biopsy samples ranging from ileum to rectum were taken from 70 male and 21 female patients (average age 47 years). Reasons for histological investigation included chronic diarrhea, cancer/adenoma screening, inflammatory bowel disease and endoscopic detection of polyps or colitis. Twelve patients were HIV-positive and two were aged 12 and 13 years. One of the children had a history of 1 month-long blood-stained diarrhea mimicking inflammatory bowel disease, which resolved upon antibiotic treatment. Five HIS positive and seven biopsies from HIS negative patients were included from a previous study [6].

1.5.2.2. Histopathological preparation and diagnosis of samples

Histopathological preparation and analysis of the intestinal biopsy samples was performed by the pathology department [1]. The appearance of the typical hematoxyphilic fringe on the brush border of the surface epithelium under light microscopy was classified as "HIS positive". Giemsa and the PAS reaction were also used by the pathologist to confirm the presence of the bacteria and exclude staining artifacts in H&E routine stain. Paraffin embedded biopsy blocks were later transferred to the Institute for Microbiology and Hygiene of the Charité University Hospital where they were deparaffinated and underwent molecular analysis as described elsewhere [1].

1.5.2.3. Oligonucleotide FISH probes

Tissue sections were hybridized with the *Brachyspira* genus-specific probe BRACHY [6] (5'-ATTAGTCCATGTTTCCAT-3') [58]. Additional hybridizing probes were used following the general guidelines for FISH mentioned above.

1.5.2.4. FISH and microscopy

For the probe BRACHY a clinical isolate of *B. pilosicoli* was used as positive control, while *Spirochaeta halophila* (ATCC29478) and *Enterococcus faecium* type strain (ATCC19434T), were used as negative controls in each FISH experiment to assure specificity of probe binding. *Brachyspira* spp. tissue colonization was categorized as focal, discontinuous or abundant, according to a scoring system adapted from Jensen et al. [31]. The invasiveness of *Brachyspira* spp. into the lamina propria and in the goblet cells was also investigated as described elsewhere [59].

1.5.2.5. PCR amplification and DNA sequencing

A semi-nested PCR was performed with a set of *Brachyspira* genus-specific primers. The first PCR targeted a 435 bp fragment of the *16S rRNA* gene using the forward 5'-GTCTTAAGCATGCAAGTC [60] and reverse 3'-GCCGAGGCTTACATTATC [61] primers. The second PCR targeted a 204 bp by using the same forward primer combined with the reverse primer 3'- AACAGGCTAATAGGCCG [60]. The amplicon was sent for sequencing to LGC Genomics [1].

1.5.2.6. Phylogenetic analysis

The *16S rRNA* gene sequences obtained from the biopsy specimens were aligned and trimmed to a uniform length of 153 nucleotides and compared with previously published *Brachyspira* spp. sequences available from GenBank using the software HUSAR 4.1. A neighbor-joining phylogenetic tree was constructed on the basis of a distance matrix corrected by the two 184 parameter model of Tamura and Nei [62, 63]. The type strains of *Brachyspira* spp. as well as an outgroup included are described elsewhere [1].

1.5.3. Methods used in the analysis of syphilis samples

1.5.3.1. Patient profile and sample characteristics

Case 1. HIV positive patient, male, 49 years of age, with symptoms typical of a B-cell lymphoma. An inguinal lymph node, examined to rule out lymphoma, showed capsular fibrosis, follicular hyperplasia, capillary endothelial hyperplasia and plasmacytosis. Although a Warthin–Starry silver stain was negative in routine investigation, a small numbers of spirochetes were visualized using immunohistochemistry.

Case 2. Male, 70 years of age, underwent surgery for phimosis and treatment for chronic wound. He had received a kidney transplant and was suffering from liver cysts, diabetes, coronary artery disease and a urinary tract infection due to *Pseudomonas aeruginosa*. A Warthin–Starry silver stain was positive and the retrospect serology confirmed the infection with *T. pallidum*.

Case 3. Male, 43 years of age, with multiple erythematous papules in the oral mucosa and gingiva, had received unsuccessful treatment with a glucocorticoid ointment for two and a half weeks. The patient was HIV positive and had received highly active antiretroviral therapy (HAART). CD4+ cell count was 575/ μL and viral load was below the detection limit. No treponemes were found in a biopsy taken from the buccal mucosa subject to histopathological diagnosis. Serologic testing confirmed the presumptive diagnosis of secondary syphilis. Therapy with benzathine penicillin led to the resolution of symptoms followed by negative serology.

Case 4. Male, 33 years of age, with cervical inflammation and tonsillitis, follicular hyperplasia of the tonsil, positive serology in serum while negative in cerebrospinal fluid. The patient had had oral sex with another man. No histopathological diagnosis was carried out.

1.5.3.2. TPALL FISH probe

T. pallidum-specific probe TPALL was designed and showed 100% homology with all *T. pallidum* subspecies 16S rRNA gene sequences available. Optimization was performed on testicular tissue sections from rabbits experimentally inoculated with *T. pallidum* subsp. *pallidum* strain Nichols, also used as positive control, kindly provided by L.Giacani . Several spirochetes and *Treponema* species were cultured [2] and fixed as described elsewhere [4] in order to be used as negative controls. The identity of the strains and positive controls was confirmed through PCR and 16S rRNA gene sequencing.

1.5.3.3. PCR amplification

A panbacterial PCR targeting the 16S rRNA was performed using the primers TPU1 and RTU8 [64] as well as primers targeting the 16S rRNA gene of *T. pallidum* as described elsewhere [65].

1.5.4. Methods used in the analysis of adhesin TP0751 (Full details on Wei-Chien et al., 2017) [3]

1.5.4.1. Animals

Four to five-week-old male C57BL/6 mice were housed at 4 mice/cage, in accordance with the most recent Guide to the Care and Use of Experimental Animals (Canadian Council on Animal Care)

1.5.4.2. Bacterial strains

All *B. burgdorferi* strains used in the experiments had been genetically modified to express green fluorescent protein (GFP) to facilitate visualization and quantification. The culturing took place following previously described guidelines. The strains used (supplementary S1 Table)[3] were: negative control strain: Non-infectious *B. burgdorferi* B31-A-derived parent strain adhesion-attenuated deficient in BBK32 protein; positive control parent strain: infectious *B. burgdorferi* expressing vascular adhesin BBK32; positive control "gain-of-function" mutant Bb-BBK32 strain: infectious *B. burgdorferi* B31-derived expressing BBK32; mutant "gain-of-function" Bb-Tp0751 strain: parent expressing TP0751 [3].

1.5.4.3. Static adhesion assay

HUVEC grown in 4-well chamber slides and coated with phenol red-free Matrigel were incubated for 12 hours with GFP-expressing *B. burgdorferi* ($1.4x10^7$) before being washed with HEPES-buffered saline, and fixed in 10% formalin buffer for quantification [3].

1.5.4.4 Flow chamber assays

HUVEC were cultivated in flow chambers, endothelia were labeled with CellMask Deep Red plasma membrane dye and washed. *B. burgdorferi* was diluted to 1 x 10^8cells/ml in 10% heat-inactivated fetal bovine serum and perfused over endothelia in flow chambers mounted in a stage-top incubator, using a syringe pump for live cell imaging and tracking [3].

1.5.4.5. Intravital microscopy

IVM for the visualization of interactions was performed in PCVs [66] of flank skin of 4- to 5-week-old anesthetized male C57BL/6 mice, 5-20 min after intravenous tail vein injection of 4×10^8 *B. burgdorferi* strains with exceptions described by Wei-Chien et al., 2017. A qPCR assay was performed to quantify the number of copies of *B. burgdorferi flaB* DNA/µl in blood from animals used for IVM. Each video was recorded for 2 minutes per PVC using a custom-built high speed multi-wavelength resonant scanner Leica TCS SP8 confocal with Leica Application Suite (LAS) software and further analyzed with Volocity software.

1.6. Results

1.6.1. Human intestinal spirochetosis samples

1.6.1.1. FISH and PCR analysis

Brachyspira spp. were visualized by FISH as a bright fringe "false brush border" on the surface of the intestinal epithelia with the BRACHY genus-specific probe in 77 out of 91 patient cases (85%) (Fig. 1 A-C) [1]. The distribution of colonization in the intestinal tract included colon (n=76), ileum and caecum (n=8), sigma and rectum (n=7). The colonization in FISH positive samples was rated in eight cases as focal (Fig. 1 C) [1] in 20 cases as discontinuous and in 49 cases as confluent (Fig. 1 A) [1]. In general, *B. pilosicoli (18/23)* samples, with a confluent false brush border, seemed to be more densely colonized than *B. aalborgi (32/53)* lineage samples. No differences in the abundance of *Brachyspira* spp. were detectable between male and female patients and the proportion of *B. aalborgi* lineage and *B. pilosicoli* was also similar for both genders. One female patient showed a double infection. Two biopsies were taken from the colon on the same day, with *B. pilosicoli* in one biopsy and *B. aalborgi* in a second one. It is the only case in the study with a double infection. However, it is not possible to rule out that a mixed infection may have been missed, because with genus-specific primers an under-represented *Brachyspira* species might not be detected by sequencing. No spirochete colonization was visualized in the biopsy samples from control patients. The two children aged 12 and 13 showed a confluent false brush border of *B. aalborgi* lineage. All patients with documented polyps, including granulation and hyperplastic polyps, were FISH positive and yielded a higher number of *B. aalborgi* (12/18). Samples with underlying adenomas (6/8) and granulation or hyperplastic polyps (13/18) showed mostly a continuous false brush border. In contrast, samples from HIV-positive patients revealed mostly focal or discontinuous colonization (8/12).

1.6.1.2. Histopathology versus molecular detection of Brachyspira spp.

FISH confirmed the histopathological diagnosis of HIS in 77 out of 91 cases. Neither FISH nor PCR found any spirochetes in the remaining 14 samples. Four of these cases showed cocci and rods on the intestinal epithelia surface suggesting a "false histopathology positive" (Fig. 2) [1]. All FISH-negative samples were also PCR negative. Among the patients with FISH-positive samples, one was diagnosed with HIS by histopathology on the first visit to the praxis and negative on the second one, with a 2-month interval, whereas FISH and PCR results were positive at both time points.

1.6.1.3. Phylogenetic analysis

PCR-amplicon sequencing confirmed the presence of *Brachyspira* spp. in 75 (82%) of the 91 patients. The phylogenetic tree (Figure 3)[1] constructed from 153 bp of the *16S rRNA* gene includes 76 sequences from this study (one patient with a double infection of *B. aalborgi* and *B. pilosicoli*. Most sequences clustered in

the *B. aalborgi* lineage that consists of three clusters as suggested by Petterson et al. [27]. The *B. aalborgi* cluster 1 contained most sequences within 49 cases, and showed a remarkable heterogeneity including two "*B. ibaraki*" phylotypes and six phylotypes with at least three mismatches to *B. aalborgi* type strain (Z22781). Together with one phylotype related to "*B. hominis*" cluster 2 and three cases of "*Brachyspira* spp. cluster 3", the *B. aalborgi* lineage accounted for a total of 53 sequences within this study; 23 cases were related to *B. pilosicoli*. The sequences showed heterogeneity with so far unreported sequences with a maximum of three mismatches. No phylotype belonged to the veterinary species *B. hyodysenteriae*.

1.6.2. Syphilis samples

1.6.2.1. TPALL probe

TPALL showed specific hybridization at a 20% (v/v) formamide concentration when all other cultivable spirochetes investigated yielded no signal. The optimization process is described elsewhere [2]. TPALL and EUB338 helped visualize and identity *T. pallidum* in testicular tissue samples of experimentally infected rabbits and all syphilis cases studied here. No unspecific probe binding was observed as confirmed by the nonsense probe NON338.

Case 1 - Lymph node. Small numbers of *T. pallidum* cells were visualized, preferentially around the blood vessels, within tissue sections of the lymph node (Fig 2) [2].

Case 2 - Preputium. Predominantly within the squamous epithelium (Fig. 3) [2], high numbers of TPALL-positive bacteria appeared to be in bundles around the cells and directed towards subepithelial stroma of the preputial tissue. Treponemes migrated deep into the squamous epithelium and superficially into the subepithelial stroma.

Case 3 - Oral mucosa. TPALL-positive bacteria were visualized in the intercellular space of the mucosal tissue (Fig. 4) [2], whereas no bacteria were visible within cells.

Case 4 - Tonsil. *T. pallidum* was predominantly found in the paracortex and the interfollicuar zone of the tonsil biopsy (Fig. 5) [2] and the hyperplastic secondary follicles with germinal centers.

1.6.2.2. PCR and analysis of DNA sequence

The species-specific PCR produced sequences between 197 and 264 bp with a 100% homology to *T. pallidum* (DQ648782) in all tissue samples.

1.6.3. Interactions of candidate adhesin Tp0751

In summary, the three strains expected to show interaction with either HUVEC or PCVs: *B. burgdorferi*

(infectious parent expressing BBK32), *B. burgdorferi* (mutant Bb-BBK32), and *B. burgdorferi* (mutant *Bb*-Tp0751) shared similar behavior in contrast to the non-infectious *B. burgdorferi* (B31-A-derived adhesion-attenuated parent strain deficient in BBK32). *Bb*-Tp0751 strain adhered to endothelia more efficiently than the adhesion-attenuated parent strain (Fig 1A) [3], in static conditions. Under physiological stress, interaction numbers were similar for *Bb*-Tp0751 and *Bb*-BBK32 (Fig 1B) [3] and greater than for the adhesion-attenuated parent strain (Fig 1B) [3]. Likewise, the dissociation rates and velocities for *Bb*-BBK32 and *Bb*-Tp0751 interactions were slower than dissociation rates and velocities for the adhesion-attenuated parent (Fig 2C and 2D) [3], indicating that both BBK32 and Tp0751 stabilized and slowed *B. burgdorferi*-endothelial interactions. Moreover, Tp0751 also promoted bacterial association with endothelia under physiological shear stress specifically by stabilizing tethered but not untethered interactions (Fig 3) [3]. In the *in vivo* experiment, Tp0751 restored vascular interactions to the adhesion-attenuated parent to levels similar to the strain expressing BBK32, and both *Bb*-Tp0751 and *Bb*-BBK32 interacted with PCVs as efficiently as the infectious control strain (Fig 5A) [3]. These results demonstrate that Tp0751 can function as a vascular adhesin in vivo.

1.7. Discussion

1.7.1. Human intestinal spirochetosis samples

HIS is routinely diagnosed by histopathology through HE-staining. Difficulties associated with the visualization of a characteristic HE-stained "false brush border" include a discontinuous or focal fringe, as well as colonization of the epithelium by bacteria, other than *Brachyspira* spp, that mimick a false brush border. Furthermore, culturing of the different *Brachyspira* species, is not commonly carried out as part of the clinical diagnostics routine due to the complex culturing media and long incubation periods required [21, 60, 67, 68]. Therefore, several molecular techniques, such as real time PCR protocols targeting the *16S rRNA* or the NADH oxidase (NOX) genes [23, 69], Multiple Locus Variable Number Tandem Repeats Analysis (MLVA) [70] and Random Fragment Length Polymorphism (RFLP) [71] have been used for the detection of spirochetes in clinical samples. Additionally, molecular testing of *Brachyspira* spp on stool samples or rectal biopsies has also been suggested [12]. However, a high risk of false positive results arises from the rich bacterial flora present in feces. Reports of healthy carriers [72] and patients where the symptoms could be attributed to underlying diseases [73, 74] should also be taken in consideration. HIS is not routinely diagnosed in microbiology laboratories and relevant phylotypes may be missed by molecular assays due to a limited number of *Brachyspira* spp. sequences available in databases mainly because studies with large patient groups are not common. In addition, PCR assays do not provide information on the *in situ* location, extension and distribution of the bacteria in patients. Therefore, the significance of the detection *Brachyspira*

spp. is still under debate and to date it is unclear how to differentiate and characterize healthy carriers, harmless colonization and infection, the latter requiring antibiotic treatment. This study showed that FISH, at the interface of histopathology and molecular biology, is a valuable tool for the diagnosis of HIS. All FISH-positive cases were confirmed through either PCR or sequencing (except for 2 cases, see details in Table 1) [1], in contrast, 14 cases first diagnosed HIS positive by histopathology, resulted to be negative when tested with FISH, PCR and sequencing. In consequence, FISH is suitable for the diagnosis and monitor of HIS in routine practice. The scoring system of Jensen et al. [31] applied here may be of use in the standardization of the diagnosis, assessing the degree of colonization in human biopsy samples.

1.7.1.1. Phylogenetic analysis

In this study, sequencing of the partial *16S rRNA* gene from human intestinal biopsies shed light into the relationship of *Brachyspira* phylotypes involved in HIS. Within the *B. aalborgi* lineage (including *"B. hominis"* and *"B. ibaraki"*), the three clusters suggested by Pettersson et al. were confirmed [27]. Most cases in this study belonged to *B. aalborgi* cluster 1, followed by *B. pilosicoli* cluster. Among the cluster 1 phylotypes two cases were identified as "*B. ibaraki*" strains [26]. This recently proposed species has so far only been reported for Japanese HIS. One case belonging to cluster 2 ("*B. hominis*"), previously proposed as a new species by Westerman et al. [23], was also reported in this study. Interestingly three sequences clustered in Petterson "*Brachyspira* spp. cluster 3". Reports on this quite distinct cluster within the *B. aalborgi* lineage are very rare [23, 27, 29]. *B. pilosicoli*, the etiologic agent of porcine intestinal spirochetosis [19], has also been described in humans [22]. Most of the samples in this study clustered close to the *B. pilosicoli* type strain as shown in previous studies [6, 23], however two samples created a new subcluster and had three mismatches to the type strain. The clinical relevance of each cluster is still unclear. In this study, it was not possible to correlate specific strains or clusters to specific symptoms or patient characteristics, as has been shown in other studies [69]. However, this might also be due to the limited clinical data available in this retrospective study. To summarize, although the cases reported here reaffirm all major HIS *Brachyspira* spp. clusters previously described, the phylogenetic diversity obtained shows to be even greater than previously reported.

1.7.1.2. Emerging trends in the etiology of HIS and clinical significance of this study

In this study 70% of HIS cases showed an infection by *B. aalborgi* lineage (n=53) and 30% by *B. pilosicoli* (n=23), which correlates to the values found in previous publications in other countries, where the abundance of *B. aalborgi* was 58-77% and *B. pilosicoli* 15-33% [12, 75]. In both mentioned studies, mixed infections were also reported. There was no significant correlation between the spirochete species and the gender, age or localization in the histological section. Previous studies have isolated *Brachyspira* spp. from several regions between the terminal ileum and rectum [13, 76]. Although the study did not systematically analyze the location of the spirochetes in the intestinal regions, the different biopsy origins did not point to major differences.

HIS was reported in 13 patients with hyperplastic or granulation polyps and in six patients with adenomas. *Brachyspira* spp. was previously described to be attached to adenomatous polyps [24, 29, 77-79] and to alter the tight junction integrity of a human epithelial colorectal adenocarcinoma cell line [80]. However, more research is needed to investigate the relationship between *Brachyspira* spp. and adenomas. Eight patients were diagnosed with a chronic gastritis of which three were *Helicobacter pylori*-associated in addition to HIS. The concurrent detection of these two bacterial species has been described before [81], although their clinical relationship remains unclear. HIS was found in four patients with ulcerative colitis as shown in previous studies [13, 82-85]. Interestingly, an improvement of the lesions in patients with ulcerative colitis has been described after treatment with metronidazole [82]. More research is needed to elucidate the clinical significance of these findings. Moreover, *Brachyspira* spp. was detected in nine HIV-positive patients. HIV was previously suggested to increase the risk of colonization by *Brachyspira* spp. [22, 86] as HIS is often found in HIV-positive patients, however no direct correlation between spirochetal colonization and immunodeficiency has been found so far [13, 14, 87]. Although the patient collective presented here is highly selective and no prospective study or epidemiology was carried out, the results support the hypothesis that HIS may be an important differential diagnosis in selected populations such as patients suffering from inflammatory bowel disease, colonic adenomas, or HIV infection as well as in MSM and possibly in children as well. Except for two patients, who harbored *Helicobacter pylori* as well as *Brachyspira* spp., no other microorganisms were found that could explain the symptoms pointing to the clinical relevance of HIS. Therefore, it is tempting to claim that the symptoms leading to endoscopy were in fact due to HIS and should raise awareness of the need to investigate for *Brachyspira* spp. colonization. Prospective studies, including molecular diagnosis, are urgently needed to investigate the clinical relevance of HIS, in identifying patients at risk.

1.7.2. Syphilis samples

Syphilis, currently re-emerging worldwide [45], might be difficult to diagnose due to its atypical clinical manifestations among patients [2]. Moreover, there are only a very few studies showing images *T. pallidum* due to constrains in cultivation and *in vivo* experimentation. This study used a *T. pallidum*-specific FISH probe to identify and localize the causative agent of syphilis within tissues in cases where clinical manifestations were inconclusive. FISH showed *T. pallidum* in lymphatic tissue of an HIV-positive patient (Case 1). These findings correlate to previous reports of the presence of *T. pallidum* infection in lymphatic tissue mimicking malignancy [88-90]. In case 2, FISH images showed *T. pallidum* in the preputial tissue of a patient initially treated surgically for phimosis, where serology was not performed on a first instance. In case 3, FISH analysis showed *T. pallidum* in an oral biopsy from a case where Warthin-Starry stain was negative, correlating to previous reports of low sensitivity of silver stains [91]. FISH showed *T. pallidum* in tonsillar

tissue (Case 4), a rare localization in primary and secondary syphilis, corresponding well to the positive histology and serology from the patient. FISH species-specific probe helped discriminate between *T. pallidum* and oral treponemes in the oral cavity [47, 48, 92-95] as well as to eliminate the suspicion of cancer in tonsils [96-99]. The findings presented here correlate to previous studies where *T. pallidum* was reported in extragenital regions [88-91, 95, 96, 100-102] and draws attention to the relevance of oral manifestations, considered to be highly contagious and a threat to dentistry personnel [102, 103]. In summary, FISH confirmed and showed through images the migratory characteristics of *T. pallidum* by providing information not accessible in research models. FISH stands as a valuable tool for understanding the pathogenesis of syphilis by investigating the invasiveness of treponemal infections and the abundance and localization of its causative agent within clinical samples.

1.7.3. Role of adhesin candidate Tp0751 in bacterial dissemination

Microbial interaction and attachment to the endothelial lining of the human vasculature through bacterial adhesion molecules is a key step in pathogen dissemination of multisystemic conditions like syphilis and Lyme disease. The identification and characterization of the molecules involved in this process are critical for the understanding of the mechanisms involved in pathogen tissue invasiveness. Moreover, the use of heterologous expression for a gain-of-function approach in related species stands as the only current tool for the study of molecular mechanisms of uncultivated bacterial species like *T. pallidum* [53, 54, 66, 104]. So far, it is possible to conclude that Tp0751 can function as vascular adhesion, in flow chambers and in mouse PCVs, which facilitates the first steps for rapid widespread to remote locations of the body. This correlates to findings reported by FISH in this dissertation where *T. pallidum* was identified and visualized in remote locations far from the suspected place of entry. Although Tp0751 may be enough for bacterial-endothelial interactions in PCVs, other adhesins and mechanisms may be necessary for bacterial interaction with other organs where shear stress may be higher. In the present study, the results of both, *in vivo* and *in vitro* experimental settings validate each other's hypotheses regarding the role of these molecules in the dissemination of *T. pallidum* and the progression of the disease, which suggest the possibility to identify additional spirochete vascular adhesins in future studies.

In summary, this dissertation explores the benefits of microscopy techniques, when combined with other molecular tools, in the understanding of infections caused by spirochetes. FISH provides insights about the invasiveness and spatial distribution of *T. pallidum* in syphilis and facilitates the accurate diagnosis of HIS. Likewise, IVM and video tracking help in the identification of virulence factors and molecules linked to motility and dissemination of the causative agents of syphilis and Lyme disease.

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3. Affidavit and declaration of contributions

I, Pablo David Rojas Mencias certify under penalty of perjury by my own signature that I have submitted the thesis on the topic “In Situ Detection, Molecular Epidemiology, and Improvement of Molecular Tools for the Understanding and Diagnosis of Infections Caused by Spirochetes” I wrote this thesis independently and without assistance from third parties, I used no other aids than the listed sources and resources.

All points based literally or in spirit on publications or presentations of other authors are, as such, in proper citations (see "uniform requirements for manuscripts (URM)" the ICMJE www.icmje.org) indicated. The sections on methodology (in particular practical work, laboratory requirements, statistical processing) and results (in particular images, graphics and tables) correspond to the URM (s.o) and are answered by me. My contributions in the selected publications for this dissertation correspond to those that are specified in the following joint declaration with the responsible person and supervisor. All publications resulting from this thesis and which I am author of correspond to the URM (see above) and I am solely responsible.

The importance of this affidavit and the criminal consequences of a false affidavit (section 156,161 of the Criminal Code) are known to me and I understand the rights and responsibilities stated therein.

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Date Signature

Declaration of Contribution

Pablo David Rojas Mencias had the following share in the following publications:

Publication 1:

Rojas P, Petrich A, Schulze J, Wiessner A, Loddenkemper C, Epple HJ, Sterlacci W, Vieth M, Kikhney J, Moter A. 2017. Distribution and phylogeny of *Brachyspira* spp. in human intestinal spirochetosis revealed by FISH and *16S rRNA*-gene analysis. Anaerobe doi:10.1016/j.anaerobe.2017.03.012.

Contribution in detail: Experiment design and literature review along with manuscript preparation. Positive and negative controls cultivation, sample collection, de-embedding and re-embedding in methacrylate, preparation and analysis with FISH, PCR sequencing and DNA analysis.

Publication 2:

Petrich A, **Rojas P**, Schulze J, Loddenkemper C, Giacani L, Schneider T, Hertel M, Kikhney J, Moter A. 2015. Fluorescence *in situ* hybridization for the identification of *Treponema pallidum* in tissue sections. Int J Med Microbiol 305:709-718. doi: 10.1016/j.ijmm.2015.08.022

Contribution in detail: Cultivation and preparation of positive and negative controls, literature review and manuscript preparation.

Publication 3:

Wei-Chien Andrew Kao, Helena Pětrošová, Rhodaba Ebady, Karen V. Lithgow, **Pablo Rojas**, Yang Zhang, Yae-Eun Kim, Yae-Ram Kim, Tanya Odisho, Nupur Gupta, Annette Moter, Caroline E. Cameron and Tara J. Moriarty. 2017. Identification of Tp0751 (Pallilysin) as a *Treponema pallidum* Vascular Adhesin by Heterologous Expression in the Lyme disease Spirochete. Scientific Reports – Nature (Sci Rep). Accepted for publication March 29th 2017. Manuscript ID: SREP-16-52098A

Contribution in detail: Methodology (intra-vital microscopy and animal handling), validation, investigation.

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Signature, date and stamp of the supervising University teacher

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Signature of the doctoral candidate

4. Printed copies of selected publications

4.1. Publication 1

Rojas P, Petrich A, Schulze J, Wiessner A, Loddenkemper C, Epple HJ, Sterlacci W, Vieth M, Kikhney J, Moter A. 2017. Distribution and phylogeny of *Brachyspira* spp. in human intestinal spirochetosis revealed by FISH and *16S rRNA*-gene analysis. Anaerobe
https://doi.org/10.1016/j.anaerobe.2017.03.012

4.2 Publication 2

Petrich A, **Rojas P**, Schulze J, Loddenkemper C, Giacani L, Schneider T, Hertel M, Kikhney J, Moter A. 2015. Fluorescence *in situ* hybridization for the identification of *Treponema pallidum* in tissue sections. Int J Med Microbiol 305:709-718.
https://doi.org/10.1016/j.ijmm.2015.08.022

4.3 Publication 3

Wei-Chien Andrew Kao, Helena Pětrošová, Rhodaba Ebady, Karen V. Lithgow, **Pablo Rojas**, Yang Zhang, Yae-Eun Kim, Yae-Ram Kim, Tanya Odisho, Nupur Gupta, Annette Moter, Caroline E. Cameron and Tara J. Moriarty. 2017. Identification of Tp0751 (Pallilysin) as a Treponema pallidum Vascular Adhesin by Heterologous Expression in the Lyme disease Spirochete. Scientific Reports – Nature (Sci Rep). Accepted for publication March 29th 2017.

Manuscript ID: SREP-16-52098A

Video files available at the Promotionskommission office of the Charité University Hospital

http://dx.doi.org/10.1038/s41598-017-01589-4

5. Curriculum Vitae

For reasons of data protection, the Curriculum vitae is not published in the online version.

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

6. Complete list of publications

Publications obtained during doctoral studies:

- **Rojas P**, Petrich A, Schulze J, Wiessner A, Loddenkemper C, Epple HJ, Sterlacci W, Vieth M, Kikhney J, Moter A. 2017. Distribution and phylogeny of *Brachyspira* spp. in human intestinal spirochetosis revealed by FISH and *16S rRNA*-gene analysis. Anaerobe doi:10.1016/j.anaerobe.2017.03.012. (Impact Factor 2015/2016: 2.424)
- Petrich A, **Rojas P**, Schulze J, Loddenkemper C, Giacani L, Schneider T, Hertel M, Kikhney J, Moter A. 2015. Fluorescence *in situ* hybridization for the identification of *Treponema pallidum* in tissue sections. Int J Med Microbiol 305:709-718. doi: 10.1016/j.ijmm.2015.08.022. (Impact Factor 2015: 3.898)
- Wei-Chien Andrew Kao, Helena Pětrošová, Rhodaba Ebady, Karen V. Lithgow, **Pablo Rojas**, Yang Zhang, Yae-Eun Kim, Yae-Ram Kim, Tanya Odisho, Nupur Gupta, Annette Moter, Caroline E. Cameron and Tara J. Moriarty. 2017 Identification of Tp0751 (Pallilysin) as a *Treponema pallidum* Vascular Adhesin by Heterologous Expression in the Lyme disease Spirochete. Scientific Reports – Nature (Sci Rep). Accepted for publication March 29th 2017 Manuscript ID: SREP-16-52098A. doi: 10.1038/s41598-017-01589-4. (Impact Factor 2017: 5.228)
- Schmiedel D, Kikhney J, Masseck J, **Rojas Mencias PD**, Schulze J, Petrich A, Thomas A, Henrich W, Moter A. 2014. Fluorescence *in situ* hybridization for identification of microorganisms in acute chorioamnionitis. Clin Microbiol Infect 20:O538-541. doi: 10.1111/1469-0691.12526 . (Impact Factor 2014: 4.575)

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Colleagues: W. Kao, H. Pětrošová, R. Ebady, Y. Zhang, Y. Kim, T. Odisho, N. Gupta, N. Zlotnikov.

Other Institutions and People

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