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Tropheryma whipplei in Africa study (TWAS)
Tropheryma whipplei and coinfections in young infants with diarrhoeal disease in Africa

Dissertation

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1 Introduction

1.1 Morbidity and mortality of children in poorly developed countries

Despite the reduction of the total number of childhood deaths since the millenium, 5,6 million children died worldwide in 2016 before the age of five years (WHO, 2017b). With approximately 500 000 child deaths per year, diarrhoeal diseases remain a leading cause for child morbidity and mortality, especially in developing countries (WHO, 2017a, UNICEF, 2016).

In 2016 with approximately 260 000 childhood deaths, the African region remained the area with the highest number of diarrhoeal deaths of children under the age of five years (WHO, 2018).

Younger age groups are more susceptible to diarrhoeal disease in general (Getachew et al., 2018) but also to death from diarrhoeal disease than older children: 72% of diarrhoeal deaths are children under the age of two years. In developing countries, on average, children under the age of three years suffer from diarrhoea three times a year (Walker et al., 2013).

1.2 Morbidity and mortality in early childhood in Ghana

Since 1998, under-five mortality in Ghana has declined by 44%, but with 60 deaths for every 1000 life births, the Millenium Developement goal of 40 deaths per 1000 life births has not yet been reached. The highest mortality rates are noted in the Northern Region with 111 deaths per 1 000 life births, the Upper Western Region with 92 deaths per 1 000 births, and the Ashanti Region with 80 deaths per 1 000 life births. 68% of all under-five deaths occur during the first year of life (Service, 2014). Pneumonia, diarrhoea, and malaria remain the leading causes of death in children under five years (Streatfield et al., 2014).

Malnutrition has improved, but 23% of the children living in Ghana are nutritionally stunted, 57% are chronically anemic, and malnutrition still contributes to one third of child mortality (unicef, 2013, UNICEF, 2011, UNICEF, 2013). Children under the age of eleven months are strongly effected by diarrhoeal disease (Anyorikeya et al., 2016) with a potential of a letal outcame (Rahman et al., 2014).

1.3 Social risk factors and consequences of diarrhoeal disease in early childhood

Hitherto, a reduced household wealth, no breast feeding practise and shared sanitary facilities have been described to be associated with diarrhoea of children in developing countries (Komarulzaman et al., 2017, Getachew et al., 2018, Thiam et al., 2017). Furthermore, adverse social conditions have been identified as a risk factor for malnutrition, reduced immune capacity, more frequent pathogen contact, and diminished physical developement (Singer, 2009).
Close contact to animals has been revealed as a potiential transmission route for various pathogens e.g. *Campylobacter spp., Toxoplasma gondii* and *Cryptosporidium* (Delahoy et al., 2018).

Each diarrhoeal episode deprives a developing body of nutrients, demanding it to recover so as not to become malnourished and ultimately vulnerable to other diseases (WHO, 2013). As demonstrated in mice models, gastrointestinal infections impair nutrient absorbtion by supporting abnormal intestinal architechture, alterations of the gastroinestinal tract (GIT) microbiota, and variations of mucosal immunity (Coutinho et al., 2008, Mondal et al., 2012, Korpe and Petri, 2012). As absorption of key nutrients is essential for neuronal development and bodygrowth, a dysfunction of intestinal absorption may lead to serious impairments of determining components of human capacity (Checkley et al., 2008, Lin et al., 2013) such as a loss of intelligence and impaired school performance and activity status (Lorntz et al., 2006, Niehaus et al., 2002, Guerrant et al., 1999).

Repeated diarrhoeal episodes have been shown to cause stress in murine models (Sudo et al., 2004), which subsequently can facilitate invasion of pathogens by dispersion of the GIT membrane. The consequential inflammatory reaction leads to alteration of the GIT microbiota and mucosal membrane and another clinical presentation of diarrhoea (Rhee et al., 2009, O'Mahony et al., 2009).
Besides an aggravation of gastrointestinal diseases, diarrhoea has also been revealed as a risk factor for other infectious diseases. In Ghana and Brazil, an increased risk for lower respiratory infection after diarrhoeal disease in children (Schmidt et al., 2009) has been reported previously. These findings are consistent

with the known risk factors for pneumonia. In 2016, pneumonia caused about 13% of the postnatal deaths of children under the age of five years worldwide (WHO, 2016, WHO, 2017b).

1.4 Etiology of diarrhoeal disease in early childhood in developing countries

Recently, the etiological profile of diarrhoeal diseases in developing countries has been analysed with extensive multi-center studies.

The comparison of eight study sites from Asia, Africa and South America confirmed that the etiology of diarrhoea varies between geographical areas and individuals of different ages. In the synopsis of all participating countries, Rotavirus, Norovirus genotype II (Norovirus II), *Campylobacter spp.,* Astrovirus and *Cryptosporidium spp.* showed the highest attributable fraction (AF) during the first twelve months of life of children. The analysis of the two African study sites delivered no congruent pathogen profile. Children with diarrhoea from South Africa showed a high impact of *Campylobacter spp.* and heat-stable enterotoxin *Escherichia coli* (ST-ETEC), whereas, in Tanzania, Norovirus II, Rotavirus and *Cryprosporidium spp*. were stronger associated with diarrhoea during the first year of life.

Primary infections were associated with diarrhoea, whereas subsequent infections did not compellingly result in clinical symptoms of diarrhoea. Additional pathogens increased the odds of diarrhoea. The protective effect of the Rotavirus vaccination was evident as Rotavius had the highest AF at study sites without a Rotavirus vaccination program but only the fifth highest AF at study centers where the vaccination program was introduced before the start of the study (Platts-Mills et al., 2015).

The impact of Rotavirus as a diarrhoeal pathogen was confirmed in another multi-site study with the highest AF for Rotavirus for children under twelve months of age at all seven study sites. All African study sites showed the second highest AF for infection with *Cryptosporidium spp*. for children under twelve months of age. The AF profile of all other pathogens varied across the different study sites (Kotloff et al., 2013).

As multiple factors affect the health of the gut, syndemic models have to be considered, too. Synergistic effects between pathogens have been confirmed e.g.,

for Rotavirus/*Giardia lamblia (G. lamblia)* or Rotavirus/*Escherichia coli* diarrhoeal diseases (Bhavnani et al., 2012). Investigation on children in early states of diarrhoeal episodes revealed significant microbiota pertubations (The et al., 2017).

1.5 Diarrhoeal pathogens in Ghana

The strong impact of Rotavirus on diarrhoeal disease was confirmed for children living in rural Ghana. The influence of an infection with malaria has been described controversingly, though the clinical manifestation of diarrhoea was rather observed in children older than twelve months of age (Ashie et al., 2017).
Additionally, Norovirus and *Shigella spp.*/enteroinvasive E. coli (EIEC) were detected as important pathogens. Coinfections with *Entamoeba dispar* (*E. dispar), Campylobacter jejuni* (*C. jejuni*) and Norovirus were described more often in the presence of *G. lamblia*. In general, infections occurring early in childhood showed a stronger assocation with diarrhoea than those in older children. *E. dispar* and *G. lamblia* were present more frequently in children of more than 12 months of age than in younger children, but however showed no association with diarrhoea (Krumkamp et al., 2015).

1.6 Tropheryma whipplei

1.6.1 Discovery, cultivation and biochemics

George Hoyt Whipple first mentioned the gram positive, rod-shaped actinobacterium in 1907 in the context of „intestinal lipodystrophy“ – Morbus Whipple (Whipple’s disease, WD). Classification of *T. whipplei* as a new species by polymerase chain reaction took until 1992 (Relman et al., 1992); the first PAS staining was performed in 1949.
Due to the long replication time of several weeks, cultivation of the pathogen had been a problem. But, since the implementation of the stable cultivation in 2000 in special mammalian cell-free (‚axenic’) growth-medium (SAM), samples from saliva, stool, cerebrospinal fluid, synovial fluid, duodenal biopsies, and strains from cardiac valves have been processed successfully (Raoult et al., 2000).

In 2003, sequencing *T. whipplei* revealed a condensed 925 938 basepair genome. The deficiency of key biosynthetic pathways, a reduced capacity for energy metabolism, and a large variety of expressed surface proteins lead to the assumption that *T. whipplei* is dependent on external nutrition and the host's immune response (Bentley et al., 2003).

1.6.2 Clinical entities

The typical clinical manifestation of an infection with *T. whipplei* was initially summarized as the rare Whipple's disease (WD). Symptoms were relatively unspecific and combinations of symptoms quiet variable. Classic Whipple's disease symptoms include weight loss (90%), arthropathy (85%), diarrhoea (75%), fever (45%), lymphadenopathy (45%), hyperpigmentation (35%), peripheral oedema (30%), cardiac murmurs (30%), occult blood loss (25%), myalgia (25%), CNS involvement (15%), chronic cough (15%), splenomegaly (15%), hepatomegaly (10%) and ascites (10%) (Marth et al., 2016). Recent investigations identified *T. whipplei* as the underlying cause for a broad range of extraintestinal manifestations that do not fulfill the criteria of a full clinical picture of WD – such as uveitis (Drancourt et al., 2008), endocarditis (García-Álvarez et al., 2016), pneumonia in immune deficient patients (Harris et al., 2007, Stein et al., 2013) and neurological affection (Blanc et al., 2011).

The bacterial load in stool samples appeared to be linked to the clinical manifestation as asymptomatic adult carriers showed a lower bacterial load than patients with WD (Schoniger-Hekele et al., 2007, Raoult et al., Fenollar et al., 2008b), whereas the bacterial load of children with enteritis was as high as found in WD patients before (Raoult et al., 2008).
Murine models confirmed an alteration of the wet weight to dry weight ratio of stool samples during an infection with *T. whipplei*. The effect was even stronger in the group with previously provoked intestinal damage as a prolonged secretion of the agent and an immunological response with antibody and cytokine production was observed there (Al Moussawi et al., 2011). Recent studies raised the hypothesis that *T. whipplei* might be considered a pathogenic agent leading to diarrhoea in childhood (Raoult et al., 2008, Raoult et al., 2010).

1.6.3 Habitat and transmission

Actinobacteria are environmental microorganisms encountered in fresh water, seawater sediments, and soil (Maiwald et al., 1998, Schoniger-Hekele et al., 2007). Occupational exposure to sewage has been shown to be one of the leading risk factors for acquiring *T. whipplei* and WD (Schoniger-Hekele et al., 2007). Studies in populations of homeless people in France suggested conditions of poor sanitation could facilitate carriage of *T. whipplei* in feces (Keita et al., 2013a).
Investigations on environmental sources in Senegal such as water, athropods, and dust samples in villages with high prevalence of *T. whipplei* showed no evidence of being a source for *T. whipplei* (Keita et al., 2013b). Few stool samples from domestic animals (i.e., chicken and goats) were tested positive in the same villages. Taking a closer look at domestic sanitary circumstances, close contact to human feces seemed to facilitate a colonization with *T. whipplei*. The theory of inter-human/fecal-oral transmission has been supported by genotype analysis previously (Keita et al., 2011).

1.6.4 Epidemiology in industrial countries

The prevalence of *T. whipplei* in fecal samples of the healthy adult population in Europe is estimated between 1–11%. Figures from a center of determination for *T. whipplei* in France reported 2,3% positive tested stool specimens of patients without WD (Fenollar et al., 2008a). Investigations in Austria introduced the hypothesis that close contact to sewage water increased the chances of excreting *T. whipplei* in feces in healthy adults (Schoniger-Hekele et al., 2007).
In a preliminary study, stool samples from French patients with and without diarrhoea were tested for *T. whipplei*. The prevalence was 3,8% for both groups, but analyzing the age of the patients revealed that *T. whipplei* was most common in children from two to four years of age with diarrhoea (15,4%) (Raoult, et al 2008). The subsequent study confirmed 15% of positively tested stool samples of French children with diarrhoea between the age of two to four years, whereas there was no positive specimen for children without gastrointestinal symptoms. Intestinal affected children with a positive stool sample for *T. whipplei* showed no chronic colonization after recovery (Raoult et al., 2010). Recent studies compared stool samples from children with an extended age spectrum from six days to six

years with and without enteritis. The case group showed 4% positive results, whereas only 1,7% positive results in the control group were reported (Fenollar et al., 2016).

1.6.5 Epidemiology in developing countries in Africa and Asia

As close contact to sewage water was found to increase the risk of acquiring *T. whipplei,* subsequent studies were designed to determine whether individuals in areas of poor sanitation show a higher prevalence of *T. whipplei (Schoniger-Hekele et al., 2007)*.

Higher prevalence was found in Gabon with 19,6% of *T. whipplei* positive volunteers. The prevalence in children decreased with age: from 40% in fecal samples of children from zero to four years down to 9,7% in samples of adults older than 20 years of age (Ramharter et al., 2014).
A similar trend was observed in two Senegalese villages as the percentage of *T. whipplei* positive tested stool samples of healthy inhabitants decreased with age from 75% (0–4 years) to 30,2% (5–10 years) to 17,4% (11–99 years). Infants suffering from diarrhoea (age 4±3,2 years), who lived in the same villages, presented a positive stool specimen for *T. whipplei* in 49% of specimens. For children under 5 years a positive serological result was associated with a positive stool sample. In contrast, every second child between the age from 5-10 years with a positive serological result was not tested positive in the stool sample (Keita et al., 2011).
In the same two Senegalese villages, an increase of *T. whipplei* colonisation with age was observed in healthy children during the first months of life: 11% of children under eight months were colonised, as well as 37% and 44% in the age between eight and 24 months and between two to ten years of age, respectively. This stratification by age suggests that initial contact with *T. whipplei* takes place between eight and 24 months of age (Fenollar 2009).
In Laos (South East Asia), healthy children from one to seven years of age were identified to be asymptomatic carriers of *T. whipplei* in feces. Here, children over the age of four years presented a significally higher rate of positive stool samples (63%) than the younger peer group (33%) (Keita et al., 2015).

Recently published data detected *T. whipplei* in 27% of stool samples from a cross-sectional study of children from two months to 15 years in Ghana with a significant increase of positive results by age. Stool specimens of children under twelve months of age with diarrhoea contained *T. whipplei* more than twice as often as children without gastrointestinal medical symptoms (Vinnemeier et al., 2016).

Synoptically, the existing data suggest that *T. whipplei* can be found ubiquitiously in the environment and that children living in ressource-poor countries may experience their first contact with *T. whipplei* in their first twelve months of life. It can be hypothesized that children in this case develope diarrhoea since they have not delevoped an adequate immunological tolerance. Later in life, *T. whipplei* is very likely to become an apthogenic intestinal commensal.
But the confirmation of this hypothesis has been impossible due to limited data about young children of under twelve months of age in previously conducted studies.

1.7 Aim of the study

The aim of the “TWAS” (*Tropheryma whipplei* in Africa study) case-control study was to assess the hypothesis that *T. whipplei* is associated with diarrhoea in infants by i) testing stool samples of children with and without acute diarrhoea for *T. whipplei* and observing the *T. whipplei* status over a period of 28 days ii) analysing the stool samples for common diarrhoeal co-pathogens and iii) investigating potential individual and socio-economic risk factors for *T. whipplei* acquisition and carriage.

2 Materials and Methods

2.1 Study area and study population

2.1.1 Republic of Ghana, Ashanti Region and Jachie-Pramso (Bosumtwi district)

With a geographical extension of 238 537 km^2 between 5° West to 1° East and 5° to 12° North, Ghana is located within the tropical belt of West Africa. The population is estimated to be 28 million. About two million of the inhabitants are settled in the main capital, Accra (UN, 2016). Since the declaration of independence in 1957, Ghana has developed toward being a progressive state in West Africa. The main export goods are oil, gold and cocoa. Education is considered one of the main political topics; the official literacy rate is about 90% of adults over 15 years. Six universities dispersed across the state offer a chance at higher education (Amt, 2016). The gross domestic product per capita amounts to 1988 US-Dollar (Worldbank, 2014). The Akan people form the major ethnic group (Amt, 2016). The estimated number of people living with HIV in Ghana totaled 270 000 in 2015 (UNAIDS, 2016). Ghana is surrounded by francophone countries with Togo in the East, Côte d'Ivoire in the West, and Burkina Faso in the North. To the South lies the Gulf of Guinea (Fig. 1).

The Ashanti region is located centrally in the middle belt of Ghana between 0° 15' West to 2° 25' West and 5° 50' North to 7° 46' North, and with an estimated population of 5 162 000 (2015) it is considered to be the most densely populated region. Most of the people belong to the Asante, the largest subgroup of the Akan. One third of all Ashanti citizen are settled in and around the regional capital, Kumasi, but in 15 of 18 districts over half of the people are residents of rural areas. About 40–50% of the population have no or only pre-school education (Government, 2016). Though English is declared as the official language of Ghana, communication in the Ashanti region is conducted predominantely in Twi (one of the Akan languages).

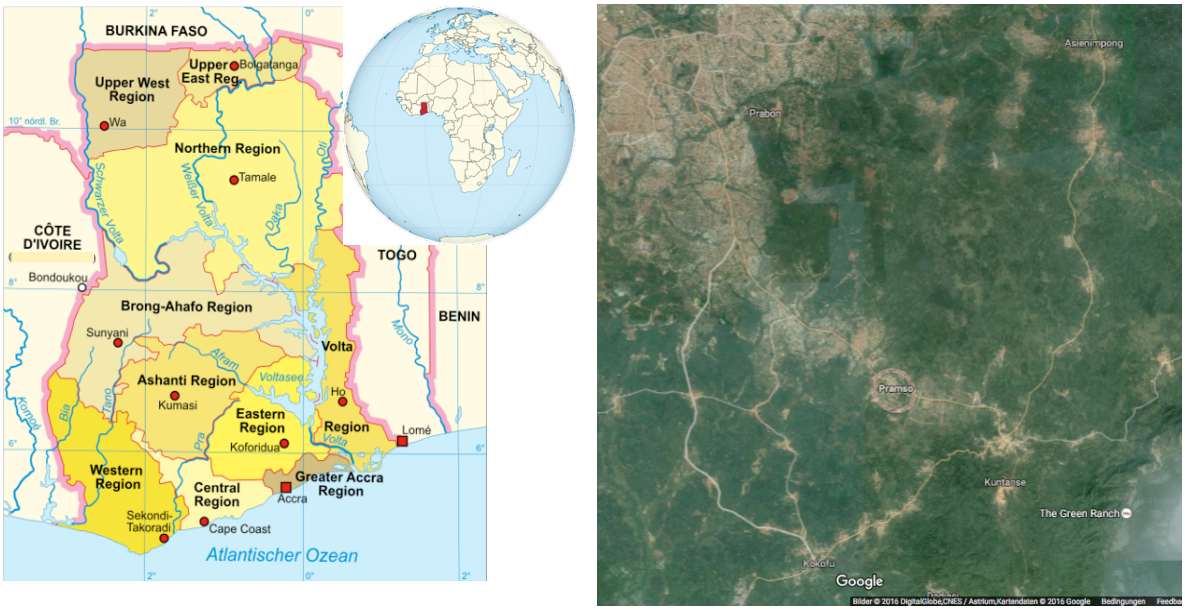


Figure 1 Study area
Left: Ghana regional and world map (https://de.wikipedia.org/wiki/Ghana)
Right: Jachie, Pramso (Bosumtwi) district from Satellite view with Kumasi on the upper left and lake Bosumtwi on the lower right (Bilder 2016 DigitalGlobe CNES/Asterium Karteidaten 2016 Google)

The Bosumtwe district lies within the Ashanti region and covers an area of 718 km^2. Following the official census of 2010 the district counts 93 910 residents, over two third settled in rural areas (Service et al., 2016). The rural village of Pramso is located approximately 25 km east of the regional capital, Kumasi.

2.1.2 The St. Michaels Catholic Hospital

Established in 1958, the St. Michaels Catholic Hospital in Pramso is a 121 bed facility with ten doctors that offers medical, surgical, and ophthalmological services; delivery facilities; a records and statistics department; a laboratory; an x-ray machine; a pharmacy department; an HIV-clinic; and an outpatient department (OPD). The focus of the clinic lies in antenatal, maternal, and pediatric care.

2.1.3 Study area

The study area was defined as a radius of 30 km around the St. Michaels Catholic hospital in the rural village of Jachie-Pramso and included rural areas as well as some peripheral municipal parts of Kumasi. The definition of this radius was necessary to ensure timely transport of stool samples to the hospital during the follow-up visits.

2.2 Study Design

2.2.1 Recruitment and case and control group selection

Inclusion criteria:
- Age of the patient between one to twelve months
- Ability of the legal guardian to understand the risks of the study, procedures, and willingness to sign the informed consent form
- Living within a radius of 30 km to St. Michaels Catholic hospital

Exclusion criteria:
- Urgent medical treatment of the patient required
- Known chronic enteric disorder
- No availability of stool sample or rectal swab within 24 hours after enrollment

Assignment to case or control group was performed according to the following criteria:

Case group:
- Diarrhoea, defined as three or more loose stools within 24 hours prior to the current visit to the OPD and a total duration of diarrhoea of no more than five days

Control group:
- No diarrhoea and no vomiting for the last seven days prior to the current visit to the OPD

A trained member of the study team screened the attending children for eligibility. After the documentation of the vital signs by the local nurses, the health care worker held an informational talk so the legal guardian would be capable of signing the informed consent form by signature or by thumbprint. The consent process was confirmed with the signature of a witness. The field worker noted personal contact details of each participating child and the legal guardian. Subsequently, the questionnaire for visit 1 (V1) was completed to acquire socio-economic data and clinical information.

The study design contained three visits. The day of enrollment was defined as visit one (V1), visit two (V2) took place on day six after enrollment, and visit three (V3) took place on day 28 after enrollment. Each patient was assigned to one of the fieldworkers who were responsible for carrying out the follow-up visits either as home visits or appointments at the hospital. The follow-up visits included a questionnaire of the child's clinical condition and a stool sample collection (Fig. 2). To increase follow-up compliance, reminder calls were performed regularly. If a child was admitted, a local nurse would perform the follow-up questionnaire and collect the sample at the children's ward.

Assuming that children during the first twelve months of life undergo an intensive physical development with a concomitant change of the environmental contact scheme, both groups were divided into three age subgroups according to the participant's age on the day of study admission (0–5 months, 6–8 months and 9–12 months).

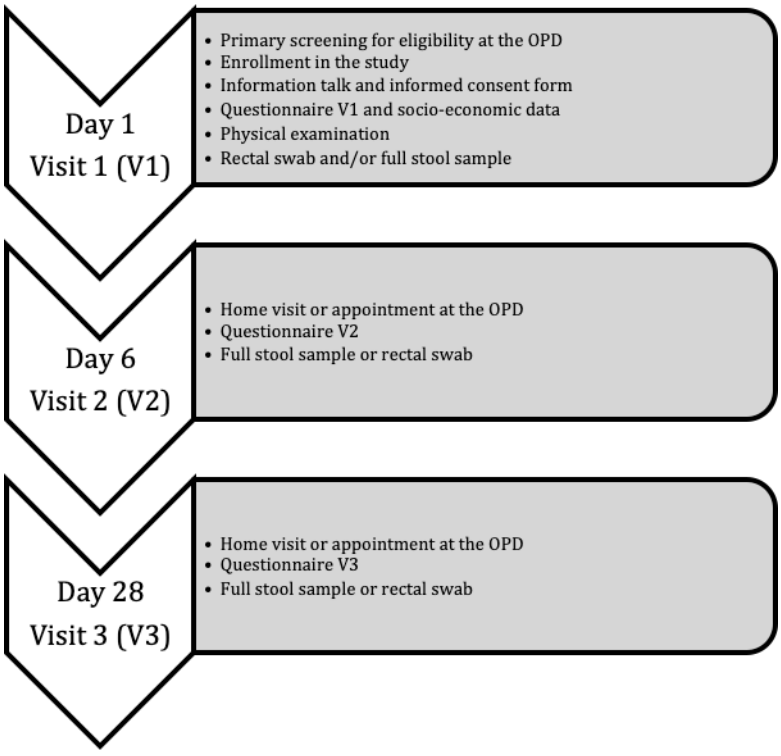


Figure 2 Flow chart study schedule

2.2.2 Clinical examination

Data collection of the medical history was based on the information given by the legal guardian and completed by a physician. Objective examination of the child was performed by a doctor. Local nurses or health care workers recorded vital signs, head circumference, temperature, and weight. In case of fever, the patients were tested for malaria via rapid test or microscopy. A physician determined the diagnosis and the treatment.

2.2.3 Sample collection

Availablility of fecal samples for all visits was essential to the underlying study design. Therefore, in addition to the collection of full stool samples, rectal swabs were taken of each child on V1. The rectal swab was performed during the visit at the OPD by trained study team members or under their supervision. Stool had to be visible on the swab. The full stool sample was collected in a labeled plastic stool container. In case a stool sample could not be provided during the visit at the hospital, a labeled stool container was handed out to the legal guardians to collect the sample within 24 hours at home. If neither a full stool sample nor a rectal swab could be provided on V1, the child was excluded from the study and subsequent follow-up would not be performed.

Legal guardians were instructed to collect a full stool sample on the dates of V2 and V3 and fieldworkers picked them up at their home. If no full stool sample could be provided within 48 hours, trained field workers performed a rectal swab. The samples were stored at 4 °C until transport to the KCCR, KNUST, Kumasi. At the laboratory, samples were stored at 4 °C if processed the same day. In case of longer storage, the sample was kept in an ultra-low-freezer at -80 °C. All samples were handled, transported and stored according to good laboratory practice.

Each follow-up visit included a questionnaire, performed by the trained local field workers or a trained local nurse. Home visits or revisits to the St. Michael hospital were arranged to collect samples and complete V2 and V3 questionnaires; reminder calls were also implemented to increase follow-up success.

2.2.4 Data handling

For each study participant an individual pseudonym (subject code) was noted on the personal data form; this form was stored separately from the remaining forms which were labeled only with the pseudonym. Only members of the study team had access to the study documents. Data were entered without personal contact details in an excel-based database on a private laptop. Only two members of the study team had access to this dataset.

2.3 Laboratory procedures

2.3.1 RNA/DNA extraction

At the laboratory, a DNA/RNA-extraction (Analytikjena innu PREP Virus DNA/RNA kit) was performed. If a stool sample and a rectal swab were available from the same visit, the stool sample was used for extraction. Each step was performed while wearing vinyl or latex gloves.

Carrier Mix stock was disolved in 1,25 ml RNAse free water and kept aliquotated at -20 °C until use. Proteinkinase K (PK) stock was solved in 1,5 ml ddH_2O and stored aliquotated at -20 °C until use. Unused Carrier Mix and PK were stored in the refrigerator immediately and not thawed more than three times. Washing solutions were prepared by adding 70 ml Ethanol to HS washing solution and 144 ml Ethanol to LS washing solution. Before each extraction, a CBV/Carrier Mix was prepared freshly by mixing 240 µl Lysis solution per sample with 12 µl Carrier Mix per sample. Per sample, approximately 50–100 µg stool were transferred into a Swab Tube, vortexed for 10 seconds, and incubated at room temperature for 15 minutes. 200 µl of this mixture were added into a 1,5 ml Eppendorf Tube and mixed with 200 µl CBV/Carrier Mix and 20 µl PK by vortexing for 10 seconds. After incubation for 15 minutes at 70 °C and a short centrifugation, 400 µl of SBS were added and mixed by another 10 seconds of vortexing. The reagent was pipetted into a Spin Filter Tube, placed in a Receiver Tube, and centrifuged for 2 minutes at 12 000 rpm. After being placed in a new Receiver Tube, 500 µl of washing solution HS was added and centrifuged by 12 000 rpm for 1 minute. After changing the Receiver Tube again, 650 µl of washing solution LS was added and centrifuged by 12 000 rpm for 1 minute. This step was repeated. Drying was conducted by

placing the Filter Tube in a new Receiver Tube, centrifuging for 5 minutes at 12 000 rpm, changing the Receiver Tube again, and centrifuging for another 2 minutes at 12 000 rpm. The Filter Tube was placed into an Elution Tube. 60 µl of preheated 70 °C RNAse free water was added and incubated for 2 minutes at room temperature. To eluate the RNA/DNA, the tubes were centrifuged at 10 000 rpm for 1 minute. The eluate was divided into two cryotubes and stored at -80 °C until transportation to the Universitätsklinikum Hamburg-Eppendorf (UKE, Germany) or until further laboratory biochemical analysis.

Before performing the PCR at the laboratories of the Institute of Medical Microbiology, Virology, and Hygiene of the UKE (Hamburg, Germany), the sample would be diluted with RNAse-free water to the total amount of 100 µl.

2.3.2 5-Colour-Quantifast-RNA/DNA-pathogen-PCR

For detection of viral, bacterial, and protozoan pathogens, professional microbiological technical assistants performed a Realtime PCR with Taqman-Oligonucleotid probe linked fluorescence dyes. The PCR included the analysis of Norovirus Genotype I (Norovirus I) and Genotype II (Norovirus II), Rotavirus, Adenovirus, Sapovirus, Astrovirus, Enterovirus, EHEC, EAEC, EPEC, ETEC*, Salmonella spp., Shigella spp./*EIEC*, Campylobacter spp., G. lamblia, Entamoeba histolytica (E. histolytica), Cryptosporidium spp*. *and T. whipplei.* IC-Assay was solved in 1100 µl Buffer TE. Primer and probes were diluted to 10 pmol. For the Global Mix 1 µl of Forwardprimer, 1 µl of Reverseprimer and 0,5 µl probe were mixed and processed following the laboratory pipetting scheme (Tab. 12 and Tab. 13 appendix). Each pathogen was verified by a positive and a negative control. 5 µl of RNA/DNA-sample and 20 µl of prepared master mix were used per well. The PCR was performed by Light Cycler II (Roche) using QuantiFast Pathogen RT-PCR+IC Kit (Quiagen) following the Stage Cycle Repeats Acquisition Temperature Time protocol with a Reverse Transcription Hold1 at 50 °C for 20 minutes, a Polymerase Activation Hold1 at 95° C for 5 minutes, a Amplification Cycling45st 95 °C for 15 seconds, a Quantification at 60 °C for 30 seconds and a cooling Hold1 at 40 °C for 30 seconds.

Real time PCR for *T. whipplei* was performed as previously published (Fenollar et al., 2008a). The target TW27 (primer and probe TW27-F TGTTTTGTACTG CTTGTAACAGGATCT; TW182 R TCCTGCTCTATCCCTCCTATCAT, TX27182-P FAM-AGAGATACATTTGTGTTAGTTGTTACA-BHQ-1) served as a screening PCR. If positive (threshold cycle (Ct) values ≤ 37) a second PCR with TW13 (primer and probe TW13-F TGAGTGATGGTAGTCTGAGAGATATGT; TW163-R TCCATAACAAAGACAACAACCAATC, TW13163P FAM-AGAAGAAGATGTT ACGGGTTG-BHQ1) was performed to increase specificity of the PCR (Fenollar et al., 2008a). Only samples positive for both targets (TW27-182 and TW13-163, both with Ct values ≤ 37) were defined as *T. wipplei* positive. Both PCRs (TW27-182 and TW13-163) were performed in 25 µl total volume using Roche LightCycler 480 II (Roche, Mannheim, Germany) the Quantifast Pathogen PCR Kit (Qiagen, Hilden Germany) and 5 µl of nucleic acid eluate. PCR2.1 carrying a single copy of the PCR product (TW27-182 or TW13-163) served as positive control in all PCR´s. A professional microbiologist performed the verification of all results.

2.3.3 Statistical analyses

According to the statistical precalculation, a sample size number of 150 children per study group was considered as appropriate.

Only patients who provided personal and clinical information in questionnaire V1 and provided a stool sample on V1 were taken into account for further analyses. For the separate analysis of the follow-up visits, only patients with a complete set of V1 data - the questionnaire and a stool sample - and a complete set of the follow-up visit's data were taken into account. For the analysis of the longitudinal development of the *T. whipplei* PCR-status, only children were eligible for whom a PCR-result and a completed questionnaire for all three visits were available.

SPSS Statistics (IBM) was used to perform the statistical analysis. Normal distributions were created according to the Kolmogorov-Smirnov test. Means were calculated and compared between groups at different time points applying statistical tests as appropriate (Student's t-test, Wilcoxon rank-test or Mann-Whitney-U-test). Proportions were compared by chi-square test or Fisher's exact

test (two-tailed), as appropriate. For analysis of interdependencies, hierarchical cluster analysis was performed by ward method with a squared Euclidean distance. A p-value of $p \leq 0,05$ was considered as statistically significant.

2.4 Ethical considerations

The case-control-study was conducted in accordance with the ethical principles of the Declaration of Helsinki and consistent with Good Clinical Practice (GCP). Ethical approval for the study was obtained from the Ethics committees of the School of Medical Science, Kwame Nkrumah University of Science and Technology (KNUST), Kumasi. The legal guardians of the participants were informed about the aim of the study in the presence of a witness and their understanding was assessed by a set of standard questions.

Participation in all phases of the study was entirely voluntary and subjects could revoke their consent at any time and without naming reasons. Informed consent was sought and granted by signature or thumbprint.

3 Results

3.1 Study population

From the 25th of August 2014 to the 31st of July 2015, 213 children were enrolled in the study. 103 control and 110 case children were considered eligible for participation. Two case children were excluded after V1 because no stool sample was provided within the defined time window. The legal guardian of a control child denied gastrointestinal symptoms in the first interview but later the legal guardian indicated vomiting for two days before attending the OPD and the infant was therefore excluded. Due to sporadic loss during transportation or storage discrepancies, not all questionnaires and not all stool samples were available for further analysis.
99 control and 105 case patients patients handed in a complete set of personal and clinical information and a stool sample on V1. Out of this collective, 90 control patients and 96 case patients provided the complete set on V2 and 87 control patients and 89 case patients provided the complete set on V3 (Fig. 3).

To be more precisely: All included patients provided a questionnaire on V1. Of these children 99 control an 105 case stool samples were available for PCR analysis. On V2, questionnaires were submitted of 93 included control patients and 102 included case patients. Stool samples of 97 control patients and 102 case stool samples were processed for PCR analysis from V2. On V3, 90 included control and 96 included case patients patients delivered a questionnaire, and 98 control and 97 case stool samples were processed (Fig. 3).

For the longitudinal analysis, 82 control patients and 83 case patients are presented in the study results with a complete set on all three visits (Fig. 4).
In detail, questionnaires of 86 control patients and 93 case patients were available on all three visits; and 89 control patients and 92 case patients provided a stool sample on all three visits (Fig. 4).

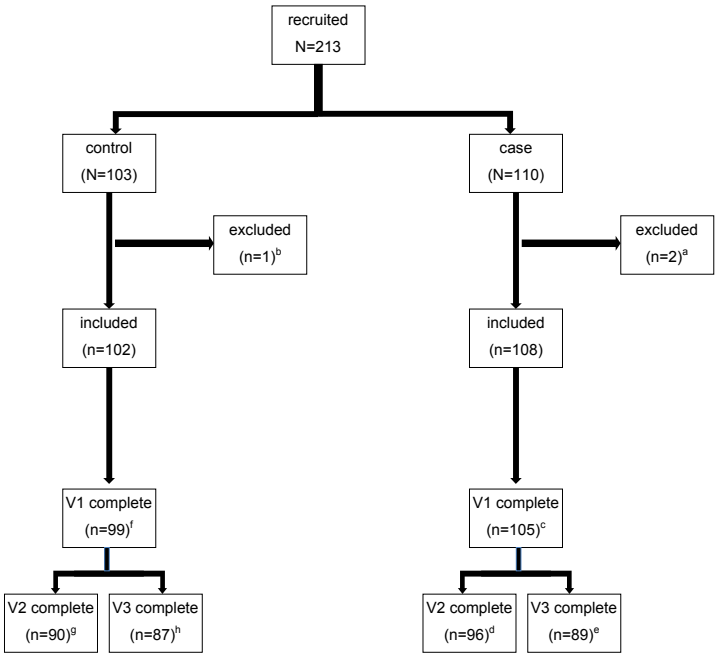


Figure 3 Patients distribution for separate analysis of the study visits; [a]=no stool provided within 24 hours after recruitment; [b]=vomiting two days before recruitment; [c]=no available stool sample (n=3), [d]=no questionnaire (n=3), no stool sample (n=3), no questionnaire and no stool sample (n=3); [e]=no questionnaire (n=5), no stool sample (n=4), no questionnaire and no stool sample (n=7); [f]=no stool sample (n=3); [g]=no questionnaire (n=4), no stool sample (n=1), no questionnaire and no stool sample (n=4); [h]=no questionnaire (n=8), no questionnaire and no stool sample (n=4);
Abbreviation: complete= questionnaire and stool sample available; N/n= number, V1= visit 1, V2= visit 2, V3= visit 3

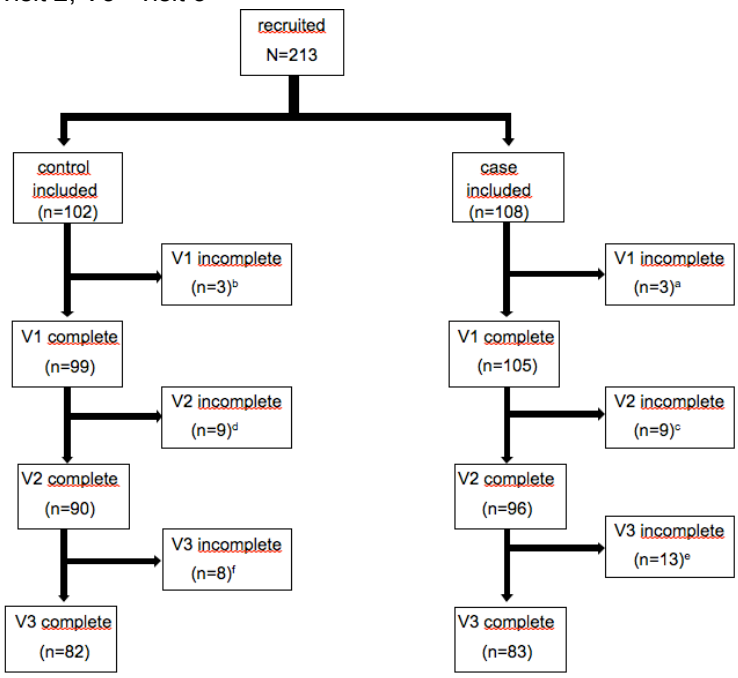


Figure 4 Patients distribution for longitudinal analysis of *T. whipplei*
Missing data: [a]=no stool sample (n=3); [b]=no stool sample (n=3); [c]=no questionnaire (n=3), no stool sample (n=3), no questionnaire and no stool sample (n=3); [d]=no questionnaire n=4, no stool sample (n=1), no questionnaire and no stool sample (n=4), [e]=no questionnaire (n=4), no stool sample (n=4), no questionnaire and no stool sample (n=5); [f]=no questionnaire (n=5), no questionnaire and no stool sample (n=3)
Abbreviation: complete= questionnaire and stool sample available N/n= number, V1= visit 1, V2= visit 2, V3= visit 3

All statistical comparison of the individual and the socio-econimic patients' characteristics refer to the baseline information, given on V1. Significant difference was detected in the mean body weight percentile (Tab. 1); case patients' mean body weight percentile was significantely lower than that of the control patients. Analysing the age subgroups, the difference of the mean body weight percentile was only replicable for children from 6–8 months (p=0,02).

The body weight percentile correlated negatively with age in both study groups for all visits separately, as well as for the patients who handed in a complete set on all three visits.

V1		**Control (n=99)**		**Case (n=105)**		
		n	**%**	**n**	**%**	**p**
Sex	Male	57	57,6	47	44,8	0,07
	Female	42	42,4	58	55,2	
Age (months)	Mean (SD)	7 (3)		7 (3)		0,6
	0–5	34	34,3	34	32,4	0,72
	6–8	37	37,4	37	35,2	
	9–12	28	28,3	34	32,4	
Weight (percentile)	Mean (SD)	36[a] (30)		28[b] (29)		0,03
	Under 3.	10	10,1	15	14,3	0,62
	3.–97.	83	83,8	84	80,0	
	Over 97.	3	3,0	2	1,9	
Nutrition	Exclusive breast feeding[c]	7,1	6,1	6	5,8	0,976
	Breast feeding and formula supplementation[d]	85	85,9	92	87,6	
	Only formula supplementation[d]	1	1,0	2	1,9	
Immunization[e]	Up to date[f]	83	98,8	83	96,5	0,621

Table 1 Individual patients' characteristics at baseline
Abbreviations: % = group percentage of available data, n=number, SD = Standard Deviation
Missing data: [a]= 3 controls; [b]= 3 cases; [c]=2 controls, 1 case;[d]= 2 controls, 1 case; [e]=15 controls, 19 cases [f]=according to the national vaccination schedule

Equal distribution between the case and the control group was statistically confirmed for most individual and socio-economic risk factors (Tab. 1) as well as both follow up visits and for those patients who handed in a complete data set including all three visits (Tab. 14, Tab. 15, Tab. 16 appendix). This equal distribution was also confirmed for the age subgroups. Comparing the case and the control group, the mode of nutrition was equally distributed. As expected, the supplementation of formula was significantely more common in older age groups.

V1		Control (n=99)		Case (n=105)		
		n	%	n	%	p
People in household[a]	Mean (SD)	9 (8,0)		10 (12)		0,75
Contact to animals	Yes	62	62,6	66	62,9	1,0
	No	37	37,4	39	37,1	
House[b]	Stone, cement, brick house	84	84,8	85	82,5	0,826
	Mud house	13	13,1	15	14,6	
	Wood house	2	2,0	3	2,9	
Floor[c]	Cement floor	41	41,4	34	33,4	0,246
	Soil floor	54	54,5	59	57,8	
	Other floor	4	4,0	9	8,8	
Bathroom[d]	Own bathroom of the family	33	33,7	37	35,2	0,883
	Shared bathroom	65	66,3	68	64,8	

Table 2 V1 Socio-economic patients' characteristics at baseline
Abbreviations: % = group percentage of available data, n= number
Missing data: [a]= 3 controls, 3 cases; [b]= 2 cases, [c]= 3 cases, [d]= 1 control

3.2 Visit 1

3.2.1 Visit 1 clinical presentation

The most frequently stated symptoms in the control group were fever, cough, and rhinorrhea. Besides diarrhoea and abdominal pain, the same symptoms were stated in the case group but less frequently. In synopsis - besides diarrhoea, vomiting, and abdominal pain – children of the case group suffered signficantely more often from tiredness and apathy (p=0,008) whereas control patients presented more often cough (p=0,01) and skin rash (p=0,002) (Tab. 3).

V1	Control (n=99)		Case (n=105)		
Symptom	**n**	**%**	**n**	**%**	**p**
Fever	74	74,7	68	64,8	0,121
Cough	64	64,6	49	46,7	0,01
Running nose	63	61,6	49	46,7	0,018
Blocked nose	52	51,5	47	44,8	0,264
Breathing difficulties	29	28,3	19	18,1	0,085
Skin rash	23	23,2	8	7,6	0,002
Eye problem	11	11,1	6	5,7	0,17
Abdominal pain	7	7,1	63	60,0	<0,001
Tiredness/Apathy	2	2,0	12	11,4	0,008
Diarrhoea	0	0	105	100,0	<0,001
Vomiting	0	0	45	42,9	<0,001

Table 3 V1 Clinical symtoms
Abbreviations: %= group percentage, n= number

104 case patients reported further details about consistency and character of the diarrhoea. The stool was described as watery in 68,3% of cases, pasty/mushy in 30,5%, and normal in 1%. 67,7% described the addition of mucus, 23,2% mentioned voluminous stools, and 2% reported bloody stools. The mean reported frequency of loose stools in the past 24 hours was 4,9 (SD 1,4). Neither the frequency (p=0,181) nor the consistancy (p=0,378) differed between the age subgroups.

The majority of control patients received the diagnosis of a respiratory tract infection (RTI), whereas enteritis was the leading diagnosis in the case group. Accounting for approximately 36%, malaria was the second most common diagnosis in both study groups (Tab. 4). The was no significant difference of general prescription of antibiotics or admission. But, specific antibiotic agents differed significantly between case and control group respective to the different types of diagnoses. Flucloxacillin and Amoxicillin/Amoxiclav were prescribed more often in the control group, whereas Metronidazole and Trimetoprim dominated in the case group (Tab. 4).

V1	**Control (n=99)**		**Case (n=105)**		
	n	**%**	**n**	**%**	**p**
Enteritis	2	2,02	86	81,9	<0,001
Malaria	34	36,2	36	36,0	1,0
RTI	69	69,7	29	27,6	<0,001
Other diagnosis	31	31,3	14	13,3	0,03
Antibiotics[a]	78	78,8	79	75,2	0,85
Amoxicillin/Amoxiclav	42	42,4	14	13,3	<0,001
Ceftriaxone	6	6,1	7	7,2	1,0
Cefuroxime	19	19,2	18	18,5	0,718
Flucloxacillin	9	9,1	0	0	0,001
Metronidazole	2	2,0	38	39,0	<0,001
Trimetoprim	1	1,0	18	18,5	<0,001
Admission[b]	9	9,1	13	12,4	1,0

Table 4 V1 Diagnosis, antibiotics and admission
Abbreviations: %= group percentage of available data, n= number, RTI=Respiratory Tract Infection
Missing data: [a]=5 cases and 5 controls;[b]= 13 controls and 20 cases
Antibiotics only listed in this table if more than 2 patients were treated with the agent.

3.2.2 Prevalence of *T. whipplei* on Visit 1, Visit 2 and Visit 3

On V1, the percentage of *T. whipplei*-positive tested children did not differ significantly between the case and the control group (p=0,832).
In the control group, 13,1% were positive for *T. whipplei* on V1, 4,4% were positive on V2, and 10,3% were positive on V3. In the case group, 11,4% had a positive result on V1, 13,5% on V2, and 20,2% on V3.

The prevalence of *T. whipplei* patients increased continuously during the time of observation in the case group, whereas in the control group the percentage of *T. whipplei* positive patients decreased on V2 – resulting in a significant difference between the two study groups. At V3 prevalence of *T. whipplei* in the control group was comparable to the prevalence at V1 (Fig. 5).

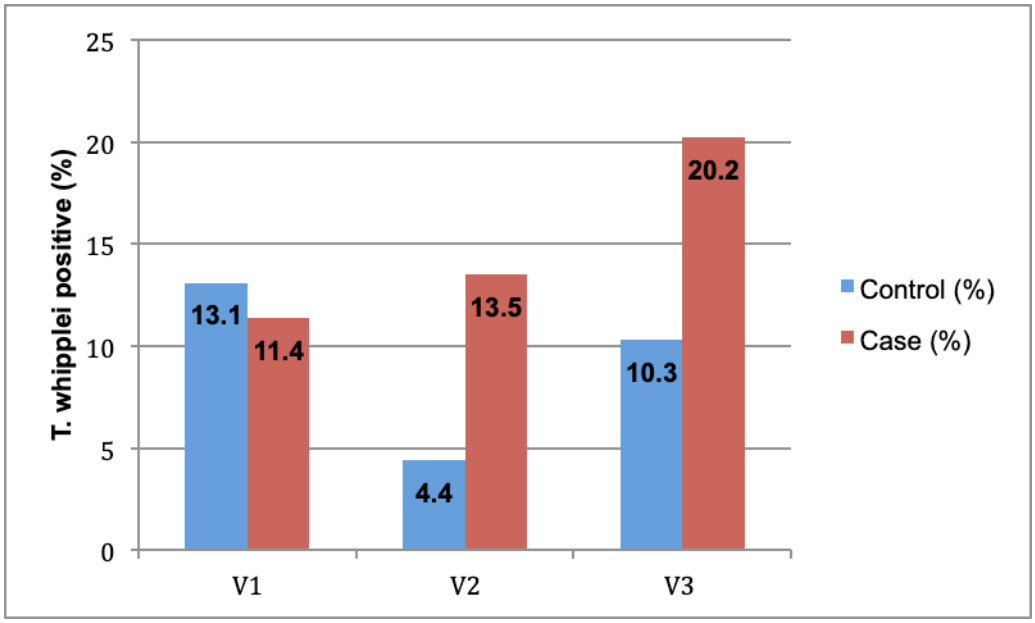


Figure 5 Percentage of *T. whipplei* positive patients on V1, V2 and V3

3.2.3 Visit 1 clinical presentation of *T. whipplei* positive patients

No single symptom applied characteristically for *T. whipplei* positive patients in the case or control group on V1. Besides diarrhea and vomiting in the case group, the most often indicated symptoms were rhinorrhea, fever, and cough in both study groups. Neither the stool frequency nor the consistency, nor the colour, nor the stool character of *T. whipplei* - positive patients were significantly different from *T. whipplei* - negative case patients (Tab. 5).

V1	Control *T. whipplei* positive (n=13)			Case *T. whipplei* positive (n=12)		
Symptom[a]	**n**	**%**	**p**	**n**	**%**	**p**
Diarrhoea	0	0	-	12	100	-
Loose stools/24h mean (SD)	-	-	-	4,8 (1,5)	0,648	0,337
Watery stools	-	-	-	8	66,7	1,0
Pasty/mushy stools	-	-	-	4	33,3	1,0
Mucoid stools	-	-	-	10	83,3	0,87
Cough	9	69,2	1,0	5	41,7	0,767
Eye problem	3	23,1	0,156	2	16,7	0,141
Abdominal pain	1	7,7	0,639	6	50	0,537
Tiredness/Apathy	0	0	1,0	3	25	0,138
Vomiting	0	0	-	7	58,3	0,084
Diagnosis[a]						
RTI	11	84,6	0,502	2	16,6	0,502
Malaria	4	30,8	0,764	1	8,3	1,0
Skin infection	2	15,4	1,0	0	0	0,289
(Gastro-) Enteritis	0	0	1,0	9	75	0,666
Antibiotics	11	84,6	1,0	7	58,3	0,223
Admission	0	0	0,590	0	0	0,055

Table 5 V1 Clinical presentation of *T. whipplei* positive patients vs. *T. whipplei* negative patients. The p-value refers to the comparision with *T. whipplei* negative patients of the respective study group.
Abbreviations: %= Percentage of *T. whipplei* positive tested control or case patients, n=number, SD= Standard Deviation
[a] = multiple symptoms /diagnoses per individual possible

The majority of *T. whipplei* positive control patients received the diagnosis RTI. Four of them were additionally treated under the hypothesis of malaria and another two *T. whipplei* positive control children received a skin infection diagnosis.

Most *T. whipplei* positive case children received the diagnosis enteritis or gastroenteritis. One of them additionally received the diagnosis malaria, and another additionally received the diagnosis RTI. One case patient with a positive PCR result for *T. whipplei* was treated under the single hypothesis of a RTI.
There was no significant difference between *T. whipplei* positive and *T. whipplei* negative tested patients concerning the given diagnosis, prescription of antibiotics, or admission in both study groups.

3.2.4 Visit 1 stool pathogens

On V1 Rotavirus (p=0,02), ETEC (p=0,04) and *G. lamblia* (p=0,01) were detected significantly more frequently in the case group. Overall, the PCR-analysis revealed a significantly higher mean number of positive pathogen (NOP) results per sample in the case group compared to control samples (p=0,003) (Tab. 6).

V1	**Control (n=99)**		**Case (n=105)**		
	n	**%**	**n**	**%**	**p**
Viruses					
Adenovirus	20	20,2	17	16,2	0,47
Astrovirus	9	9,1	15	14,3	0,28
Enterovirus	36	36,4	41	39,0	0,77
Norovirus I	3	3,0	2	1,9	0,675
Norovirus II	5	5,1	12	11,4	0,13
Rotavirus	2[a]	2,0	11[b]	10,5	0,02
Sapovirus	4	4,0	10	9,5	0,17
Bacteria					
T. whipplei	13	13,1	12	11,4	0,83
Campylobacter	18	18,2	28	26,7	0,25
EAEC	44	44,4	53	50,5	0,40
EHEC	10	10,1	11	10,5	1,0
ETEC	20	20,2	35	33,3	0,04
EPEC	34	34,3	40	38,1	0,66
Salmonella	1	1,0	0	0	0,49
Shigella/EIEC	3	3,0	7	6,7	0,33
Protozoa					
Cryptosporidium	5	5,1	13	12,4	0,08
Entamoeba	0	0	1	1	1,0
G. lamblia	2[c]	2,0	12[d]	11,4	0,01
Negative for all agents	14	14,1	7	6,7	0,11
Positive for any agent	85	85,9	98	93,3	0,106
Monoinfection	16	16,2	18	17,1	1,0
Pathogens/sample mean n (SD)	2,3 (1,7)		3,1 (1,8)		0,003

Table 6 V1 Pathogens, monoinfections, number of pathogens per sample
[a]=2 months, 6 months; [b]=3 children 1-3 months, rest 6-10 months; [c]= 7 months, 10 months; [d]=7-12 months; Abbreviations: %=group percentage, n= number, SD=Standard Deviation

Repeating the comparision of the NOP between the case and the control group on the level of the age subgroups, only revealed a significant higher NOP for case patients older than 6 months ($p_{6\text{-}8\ months}$=0,037; $p_{9\text{-}12\ months}$=0,016). Additionally,

samples without any pathogen were found less frequently in case group children older than 9 months (p=0,037), compared to control group children of the equivalent age (Fig. 6).

The age and the NOP showed a significant positive correlation in both study groups (p_{case}<0,001; $p_{control}$=0,017). Additionally, in the control group monoinfection was detected significantly more often in children of 6–8 months (p=0,018) than in the oldest age subgroup (Fig. 6). As described for the case and control group in total above (Fig. 5), also on the level of the age subgroups there was no significant difference of the occurrence of *T. whipplei* between the equivalent age subgroups (Fig. 6).

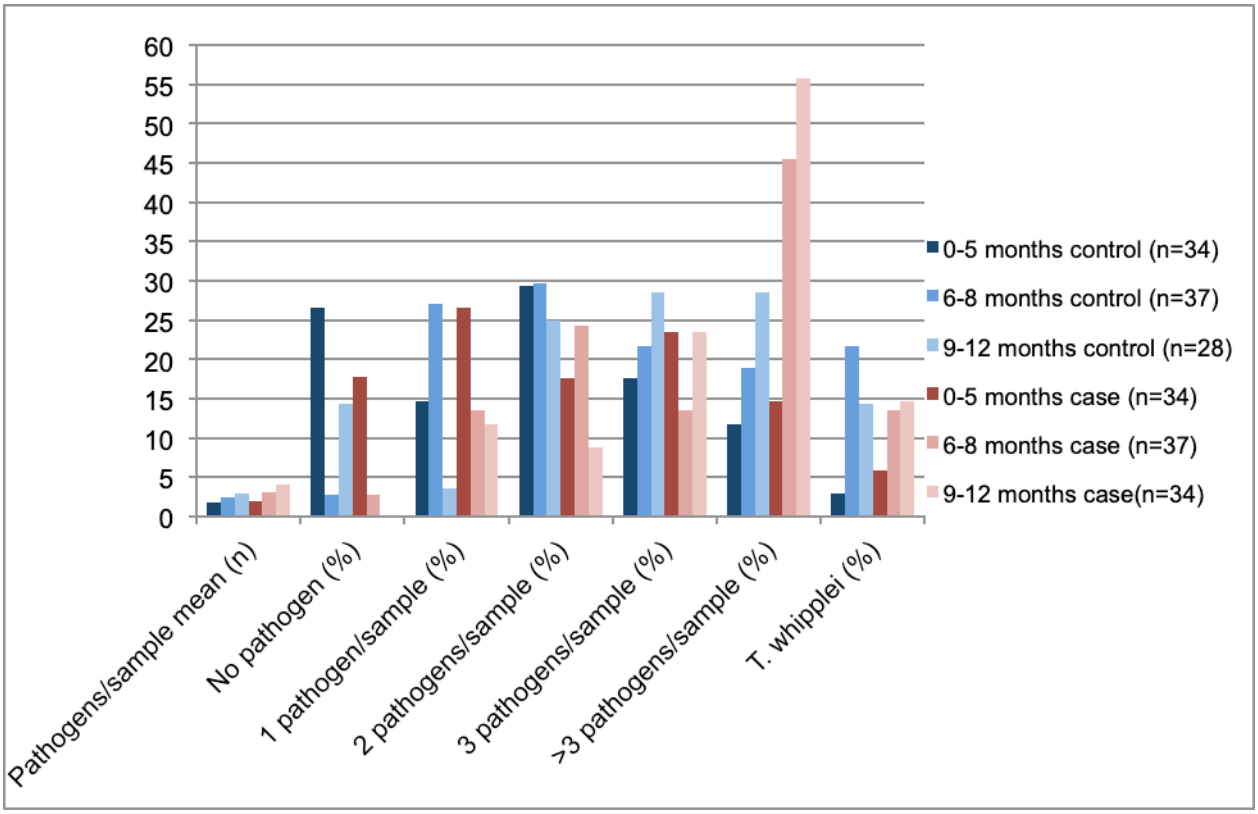


Figure 6 V1 Numbers of pathogen per sample and *T. whipplei* - Distribution over age subgroups. Abbreviaton: n= number, %= percentage of age subgroup

3.2.5 Visit 1 *T. whipplei* copathogenity and the holistic pathogen profile

On V1, the NOP was significantly higher for *T. whipplei* positive samples compared to *T. whipplei* negative samples for case patients (NOP_{TW+}=3,9; NOP_{TW-}=2,8; p=0,023) but not for control patients (NOP_{TW+}=2,4; NOP_{TW-}=2,2;

p=0,782) (Fig. 7). There was no *T. whipplei* monoinfection detectable for case patients and only one *T. whipplei* monoinfection on V1 of a control patient.

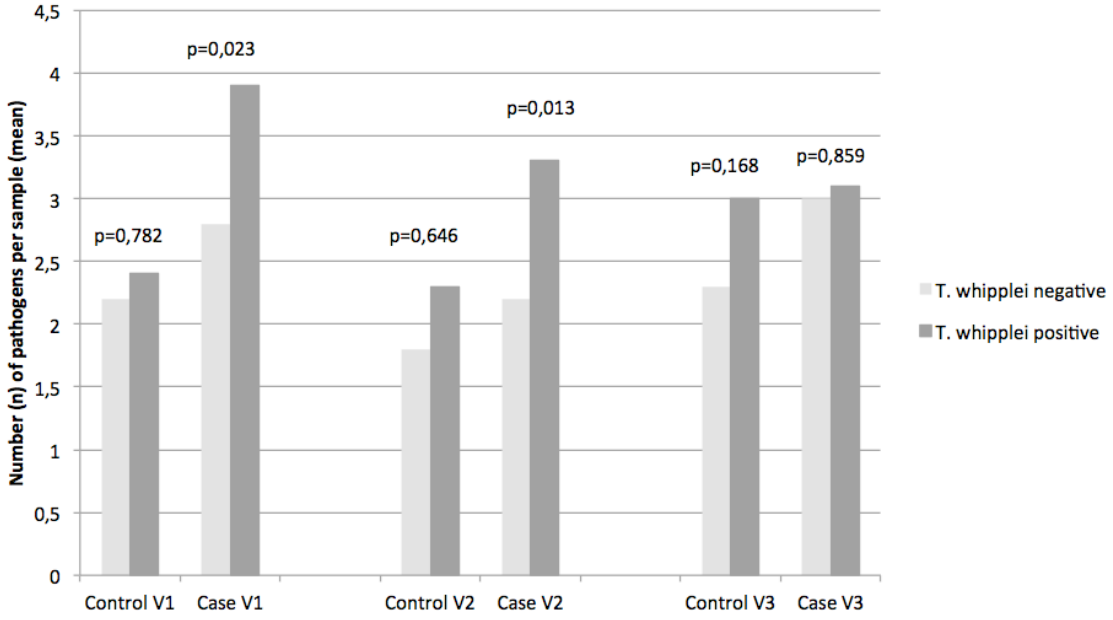


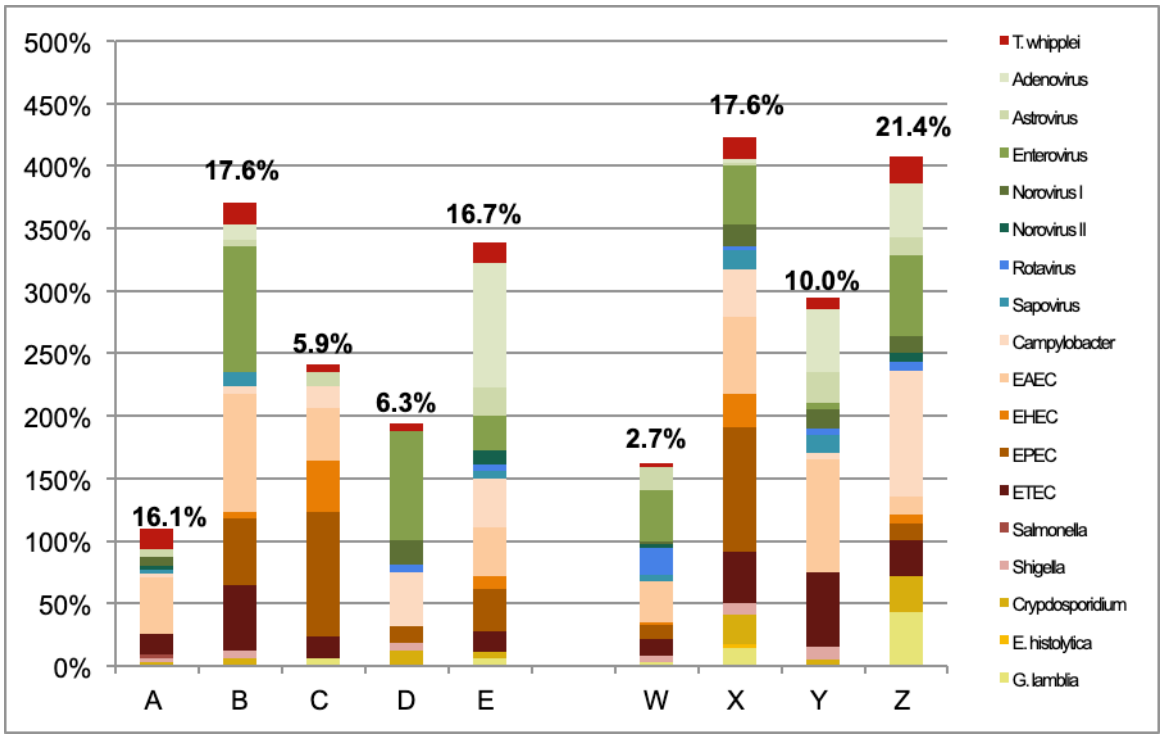
Figure 7 Number of pathogen per sample of *T. whipplei* positive versus *T. whipplei* negative samples. Counted were all positive PCR-results except for *T. whipplei.*

On V1, the only significant pairwise co-occurrence with *T. whipplei* that could be demonstrated was the combination of *T. whipplei and* Norovirus II in case children; Children with Norovirus II infection had about a nine times higher chance of testing positive for *T. whipplei* on the same visit compared to children that did not test positive for Norovirus II (OR 8,78 (95%CI 2,2-35,0), p=0,004).

In order to picture and compare the co-occurrence of multiple pathogens, a hierachic cluster analysis based on the similarity of the patients' pathogen results on V1 was performed. According to the resulting dendrogramm (Fig. 13 appendix), the individual patients were assigned to one of five control (A-E) or one of four case (W-Z) pathogen clusters.

As the numbers of patients per cluster varied from 14-37 patients per cluster, the percentage of patients who were tested positive for a specific pathogen were used to compare the cluster to each other. The colums of Fig. 8 picture the sum of percentages of all positive tested pathogens in the respective cluster. Therefore, the y-axis exceeds 100%.

The percentages of *T. whipplei* positive patients of the cluster are indicated on top of the colums. The NOP per sample of the patients varied between the clusters from 1,1 to 3,7 in the control and 1,6 to 4,2 in the case group. *T. whipplei* occurred equally distributed in each control cluster (p=0,751) and each case cluster (p=0,127) (Fig. 8).



n	31	17	17	16	18		37	34	20	14
NOP	1,1	3,7	2,4	1,9	3,4		1,6	4,2	3	4,1
Age	5,7	5,7	7,9	7,4	6,8		5,4	7,6	6,7	8,3

Figure 8 V1 Cluster analysis of the holistic pathogen profile
Accordig to the similarities of their pathogen profile, the control group was separated into five cluster (A-E), the case group into four cluster (W-Z). Indicated are the percentages of positive tested patients of each pathogen in the individual cluster. Thus, each colum represents the sum of pathogen percentages in the respective cluster. Above the colums, the percentage of positive for *T. whipplei* tested patients of the respective cluster are indicated. The table underneath shows basic characteristics of the cluster as the number of patients per cluster, the mean number of pathogens per sample and the mean age (months).
Abbreviations: n=number of patients per cluster, NOP=mean number of positive pathogens per sample

3.3 Follow-up visits Visit 2 and Visit 3

3.3.1 Follow-up visits – clinical presentation

On both follow-up visits, for the majority of children no clinical symptoms were reported by their legal guardians. Ten case children suffered from diarrhoea on V2 continously since inclusion. There were no reports of diarrhoea on V3 in cases and for only one child of the control group (Tab. 7).

V2	**Control (n=90)**		**Case (n=96)**		
Symptom	**n**	**%**	**n**	**%**	**p**
Diarrhoea	0	0	10	10,4	0,002
Fever	2	2,2	3	3,1	1,0
Cough	2	2,2	1	1,1	0,613
Running nose	0	0	5	5,2	0,06
Blocked nose	1	1,1	3	3,1	0,622
Breathing difficulties	3	3,3	0	0	0,111
Abdominal pain	1	1,1	3	3,1	0,622
Vomiting	1	1,1	1	1,0	1,0
Tiredness/Apathy	1	1,1	0	0	0,484
Eye problem	1	1,1	3	3,03	0,622
No symptom	83	92,2	77	80,2	0,021
V3	**Control (n=87)**		**Case (n=89)**		
Symptom	**n**	**%**	**n**	**%**	**p**
Diarrhoea	1	1,1	0	0	0,494
Fever	2	2,3	1	1,1	0,619
Running nose	2	2,3	1	1,1	0,619
Blocked nose	1	1,1	0	0	0,494
Breathing difficulties	1	1,1	0	0	0,494
Abdominal pain	0	0	1	1,1	1,0
Tiredness/Apathy	1	1,1	0	0	0,494
No symptoms	80	92	86	96,6	0,209

Table 7 V2 and V3 Clinical symptoms
Abbreviations: % =group percentage, n= number

3.3.2 Follow-up visits – stool pathogens

On V2 the NOP per sample remained higher in the case group compared to the control group (p=0,023) on V2 (Tab. 8). But on the level of the age subgroups, the NOP was significantly higher for case patients only at the age of 6–8 months (p=0,009) compared to the equivalent control age subgroup (Fig. 9).

In contrast to V1 (when in both study groups the NOP correlated significantly with the age), the NOP correlated significantly with age only in the case group (p_{case}=0,034; $p_{control}$=0,074). However, control children of 9–12 months had significantly fewer monoinfections compared to control children of 0–5 months (p=0,013) and control children of 6–8 months (p=0,003). The NOP of children from 6–8 months exceeds three pathogens less frequently in the control than in the case group (p=0,017) (Fig. 9).

In general, the analysis of the single pathogen prevalence revealed significantly more positive results of *T. whipplei* (p=0,041) and EHEC (p=0,019) in the case group compared to the control group (Tab. 8). These finding could not be confirmed in the level of the age subgroups (Fig. 9).

V2	**Control (n=90)**		**Case (n=96)**		
	n	**%**	**n**	**%**	**p**
Viruses					
Adenovirus	14	15,6	19	19,8	0,565
Astrovirus	6	6,7	9	9,4	0,595
Enterovirus	37	41,1	38	39,6	0,882
Norovirus I	1	1,1	4	4,2	0,37
Norovirus II	6	6,7	11	11,5	0,314
Rotavirus	3	3,3	6	6,3	0,499
Sapovirus	4	4,4	7	7,3	0,539
Bacteria					
T. whipplei	4	4,4	13	13,5	0,041
Campylobacter	13	14,4	16	16,7	0,692
EAEC	32	35,6	43	44,8	0,232
EHEC	1	1,1	9	9,4	0,019
ETEC	15	16,7	17	17,7	1,0
EPEC	25	27,8	25	26,0	0,869
Salmonella	0	0	0	0	-
Shigella/EIEC	1	1,1	7	7,3	0,066
Protozoa					
Cryptosporidium	4	4,4	11	11,5	0,11
Entamoeba	0	0	0	0	
G. lamblia	1	1,1	4	4,2	0,37
Negative for all agents	15	16,7	12	12,5	0,275
Positive for any agent	75	83,3	84	87,5	0,533
Monoinfection	29	32,2	23	24,0	0,253
Pathogens/sample mean (SD)	1,9 (1,4)		2,5 (1,8)		0,023

Table 8 V2 Pathogens, monoinfections and numbers of pathogen per sample
Abbreviations: %=group percentage, n=number, SD=Standard Deviation

Comparing the equivalent age subgroups of the case and the control group did not show any statistically significant difference among the age subgroups regarding the occurrence monoinfections, or number of samples without a positive PCR result (Fig 9).

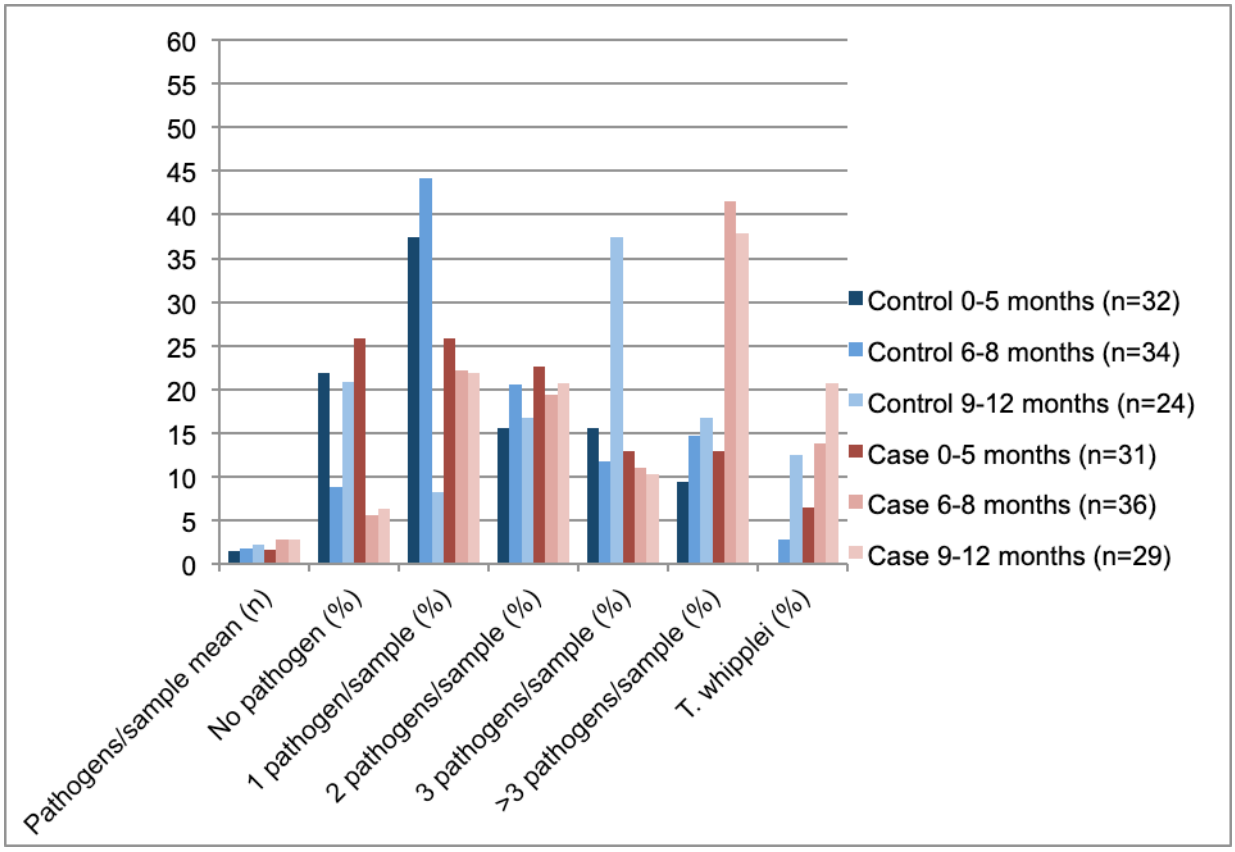


Figure 9 V2 Numbers of pathogen per sample and *T. whipplei* – Distribution over age subgroups. Abbreviaton: n= number, %= percentage of age subgroup

Also on the second follow-up visit (V3), more pathogens were found in samples of case children compared to the control patients' samples (p=0,003) (Tab. 9). Comparing the equivalent age subgroups between the case and control groups revealed that the difference of the NOP was significant for the youngest (p=0,024) and the middle age subgroup (p=0,025), but not significant for children from 9–12 months (Fig. 10).

In contrast to V2, the NOP correlated significantly with age only in the control group (p=0,009). However, this was not seen in the case group (p=0,101). Analogically, within the control group, the NOP was significantly lower in the youngest age subgroup, compared to the two other age subgroups (p=0,028). The youngest age subgroup also showed no positive PCR-result significantly more often when compared to the older age subgroups (p=0,034). But there was no

significant difference detectable concerning monoinfections (p=0,941). In contrast, within the case group, no significant difference was detectable between the age subgroups concerning samples without any detected pathogen, monoinfections, or the NOP (Fig. 10).

T. whipplei occurred equally in the case and the control group in total (Tab. 9) – as well as on the level of the age subgroups (Fig. 10).

In general, significantly more positive results in the case group were detectable for Astrovirus, *Campylobacter spp.* and *Cryptosporidium* (Tab. 9).

V3	**Control (n=87)**		**Case (n=89)**		
	n	**%**	**n**	**%**	**p**
Viruses					
Adenovirus	16	18,6	26	29,2	0,112
Astrovirus	1	1,1	8	9,0	0,034
Enterovirus	43	49,4	38	42,7	0,45
Norovirus I	1	1,1	2	2,2	0,62
Norovirus II	10	11,5	10	11,2	1,0
Rotavirus	0	0	5	5,6	0,059
Sapovirus	8	9,2	9	10,1	1,0
Bacteria					
T. whipplei	9	10,3	18	20,2	0,094
Campylobacter	14	16,1	27	30,3	0,032
EAEC	37	42,5	51	57,3	0,07
EHEC	13	14,9	13	14,6	1,0
ETEC	15	17,2	22	24,7	0,268
EPEC	37	42,5	38	42,7	0,982
Salmonella	0	0	2	2,2	0,497
Shigella/EIEC	5	5,7	2	2,2	0,275
Protozoa					
Cryptosporidium	1	1,1	12	13,5	0,002
Entamoeba	0	0	0	0	-
G. lamblia	6	6,9	7	7,9	1,0
Negative for all agents	10	11,5	4	4,5	0,075
Positive for any agent	77	88,5	85	95,5	0,101
Monoinfection	16	18,4	11	12,4	0,301
Pathogens/sample mean (SD)	2,5 (1,6)		3,3 (1,7)		0,003

Table 9 V3 Pathogens, monoinfections and number of pathogens per sample
Abbreviations: %= group percentage of control or case group, n= number, SD=Standard Deviation

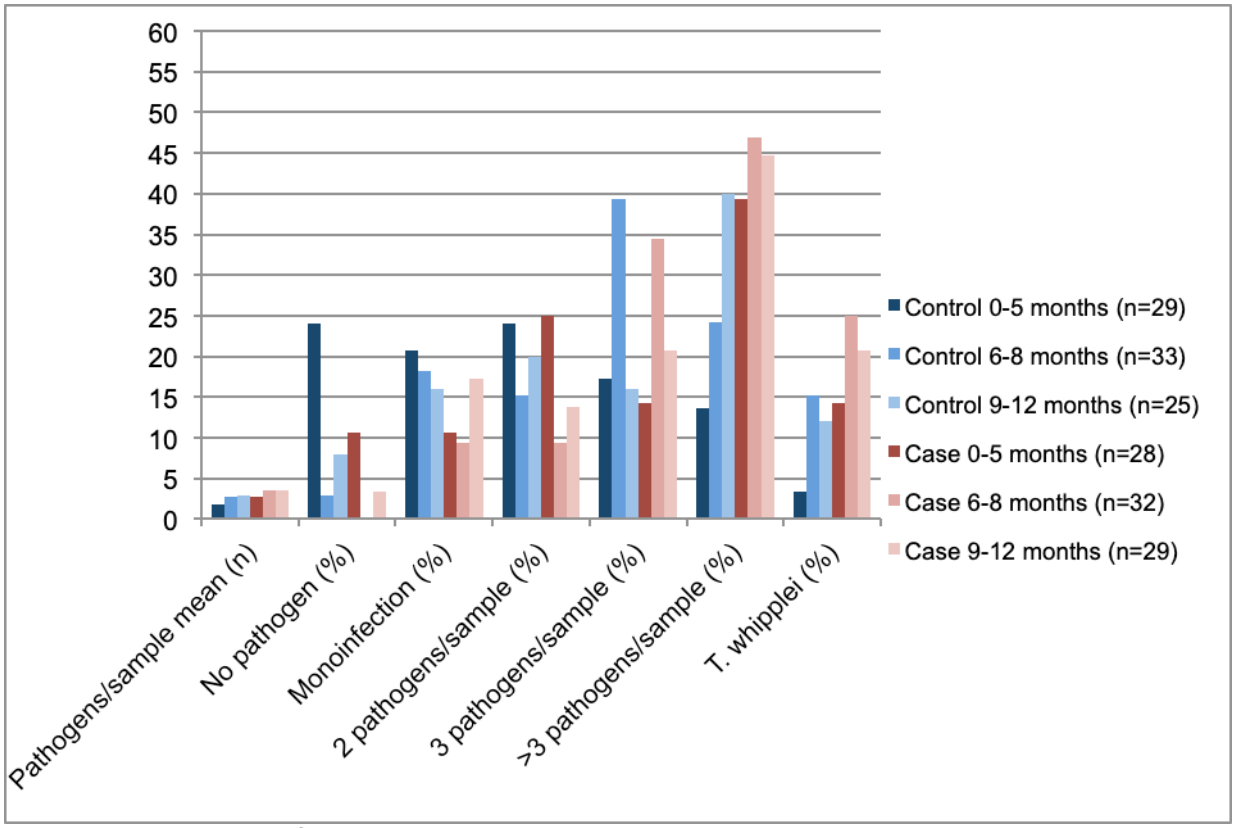


Figure 10 V3 Numbers of pathogen per sample and *T. whipplei* – Distribution over age subgroups. Abbreviations: % = Percentage of the age subgroup, n=number

The general prescription of antibiotics as well as of specified agents such as Amoxicillin/Sulbactam or Metronidazole did not influence the *T. whipplei* status significantly on either follow-up visit in either study group.

3.3.3 Follow-up visits – clinical presentation of *T. whipplei* positive patients

Case patients with a *T. whipplei* positive status on V2 suffered significantly more often on V2 continuously from diarrhoea since the initial visit at the hospital than *T. whipplei* negative case patients (p=0,028). This relation was not confirmed for any other pathogen (Tab. 10).

Control children with a positive result for *T. whipplei* on V2 did not present any symptoms. On the contrary, six out of thirteen *T. whipplei* positive case patients reported one or more symptoms on V2, which was significantly more than *T. whipplei* negative case patients (p<0,001). The combination of symptoms on V2 (if more than one were indicated) was individual for each *T. whipplei* positive case patient on V2.

V2	Control *T. whipplei* positive (n=4)			Case *T. whipplei* positive (n=13)		
Symptom	**n**	**%**	**p**	**n**	**%**	**p**
Diarrhoea	0	0	-	4	30,8	0,028
Fever	0	0	1,0	1	7,7	0,357
Running nose	0	0	-	1	7,7	0,525
Blocked nose	0	0	-	2	15,4	0,047
Breathing difficulties	0	0	1,0	0	0	-
Cough	0	0	1,0	0	0	1,0
Eye problem	0	0	1,0	1	7,7	0,357
Vomiting	0	0	1,0	0	0	1,0
Abdominal pain	0	0	1,0	0	0	1,0
Tiredness/Apathy	0	0	1,0	0	0	-

Table 10 V2 Clinical presentation of *T. whipplei*
Abbreviations: %= group percentage of *T. whipplei* positive tested control or case patients, n=number

On V3, the constellation of reported symptoms aligned for *T. whipplei* positive and *T. whipplei* negative patients in both the case and control group. One *T. whipplei* positive control patient who reported diarrhoea on V3 was newly positive for Rotavirus and Sapovirus on V3, too.

3.3.4 Follow-up visits – *T. whipplei* copathogenity and the holistic pathogen profile

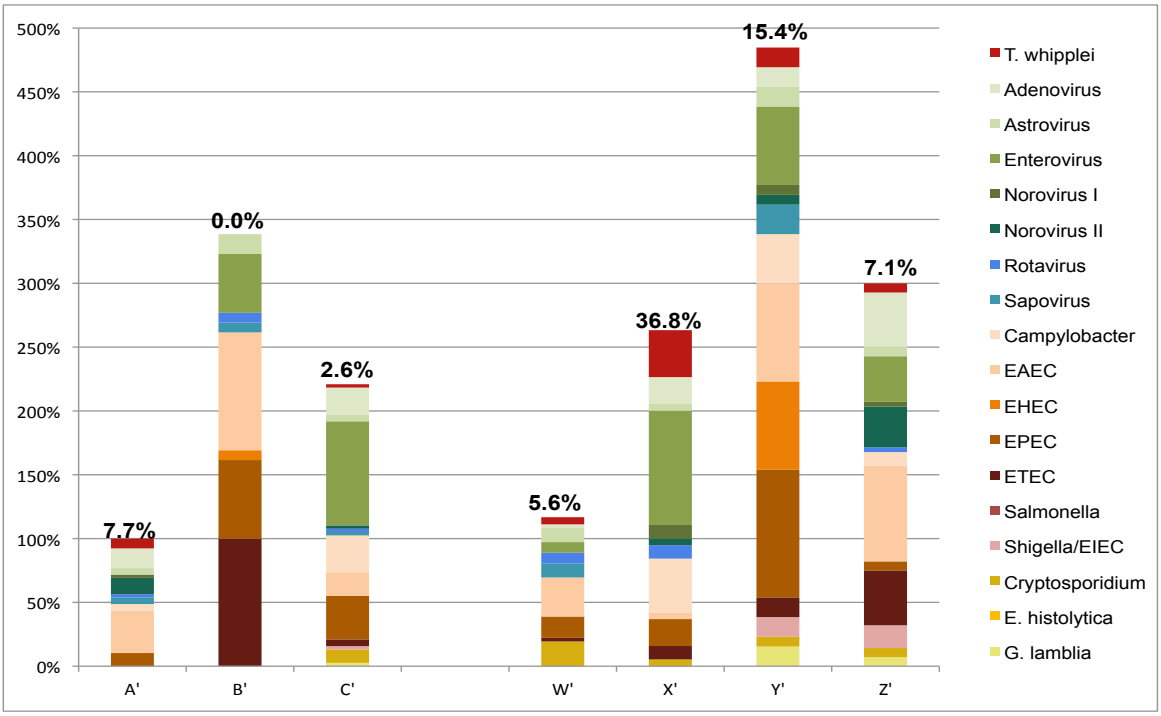
As observed on V1 before, the NOP of *T. whipplei* positive samples compared to *T. whipplei* negative samples on V2 was significantly higher for case patients (NOP_{TW+}=3,3; NOP_{TW-}=2,2; p=0,013) but not for control patients (NOP_{TW+}=2,3; NOP_{TW-}=1,8; p=0,646) (Fig. 7). Comparing the equivalent age subgroups of the case and the control group regarding the NOP of *T. whipplei* positive and negative tested patients showed no significant difference.

On V2, the pairwise analysis of each pathogen with *T. whipplei* indicated higher odds for case children with a *Campylobacter spp.* positive PCR-result to be positive for *T. whipplei* as well (OR 4,09 (95% CI 1,13-14,79), p=0,039)), whereas the OR of 2,06 (95%CI 0,2-21,42), p=0,47) in the control group was not significant.

On V3 - contrary to the previous observations - there was no significant difference detectable concerning the NOP between *T. whipplei* positive and negative samples in either the case (NOP_{TW+}=3,1, NOP_{TW-}=3; p=0,168) or the control group

(NOP_{TW+}=3, NOP_{TW-}=2,3; p=0,859) (Fig 7). Nor was there a significant combination of defined pathogens acting as a co-infection with *T. whipplei* on V3.

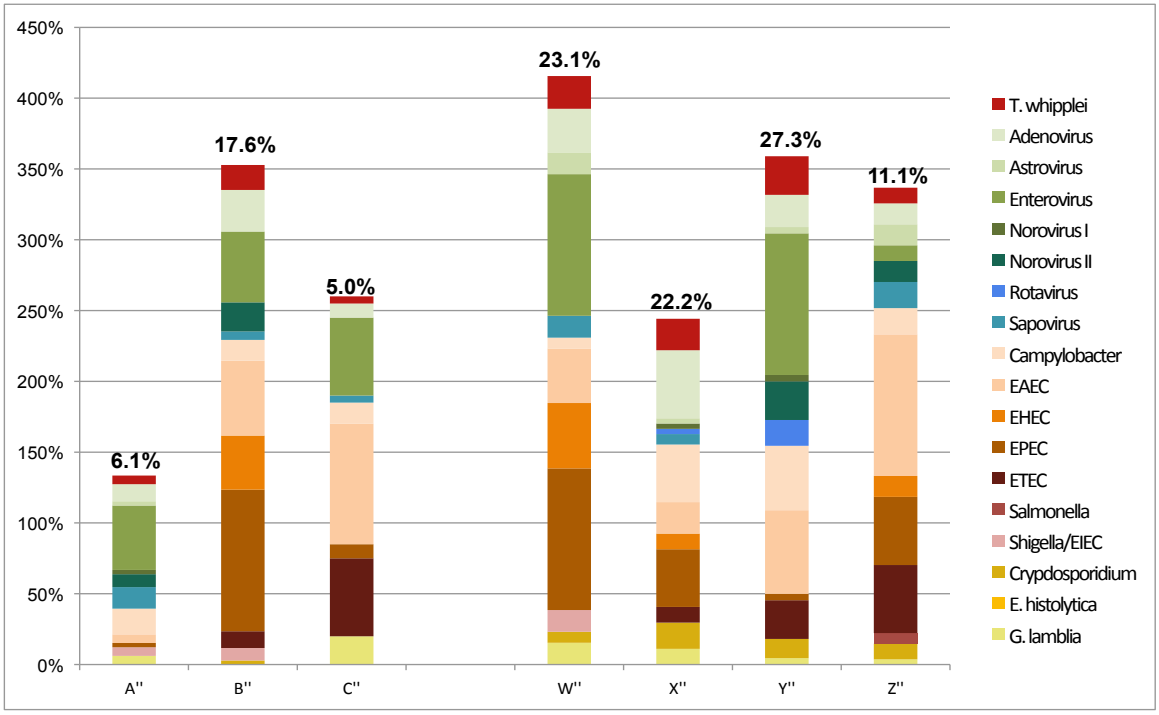
As described above, in order to analyse the constellation of pathogens around *T. whipplei,* according to the dendrogramms (Fig. 14 and Fig. 15 appendix), patients were sorted into one of three control clusters (A', B', C') or one of four case clusters (W', X', Y', Z') for V2 (Fig. 11) and for V3 into three control (A'', B'', C'') and four case cluster (W'', X'', Y'', Z'') (Fig. 12). The hierarchic cluster analysis to obtain the dendrogramms was conducted separately for each visit.
On V2, *T. whipplei* appeared with a fraction of 36,8% in X', beeing statistical significant more often than in the other pathogen clusters (p=0,011). Whereas in the control group, *T. whipplei* occurrence was distributed equally (p=0,657) (Fig. 11).



n	39	13	38		36	19	13	28
NOP	1,0	3,4	2,2		1,2	2,6	4,8	3,0
Age	6,3	4,6	7,2		6,6	6,2	6,9	7,1

Figure 11 V2 Cluster analysis of the holistic pathogen profile. According to the similarities of the pathogen profile on V2, the control group was separated into three (A'–C') and the case group into four (W'–Z') pathogen cluster. Indicated are the percentages of positive tested patients for each pathogen in the cluster. Thus, each colum represent the sum of percentages of the respective cluster. Above the colums, the percentage of positive for *T. whipplei* tested patients of the respective cluster are indicated. The table underneath shows basic characteristics of the cluster as the number of patients per cluster, the mean number of pathogens per sample and the mean age (months). Abbreviations: n=number, NOP=mean number of positive pathogens/sample

On V3, *T. whipplei* was equally distributed over all clusters in both study groups ($p_{control}$=0,875; p_{case}=0,524). The NOP varied from 1,4 (A'') to 3,5 (B'') in the control group and from 2,4 (X'') to 4,2 (W'') in the case group (Fig. 12).



n	33	34	20		13	27	22	27
NOP	1,4	3,5	2,6		4,2	2,4	3,6	3,4
age	6,5	7,5	5,4		7,2	6,7	5,9	7,3

Figure 12 V3 Cluster analysis of the holistic pathogen profile. According to the similarities of the pathogen profile the control group was seperated into three (A''-C'') and the case group into four (W''-Z'') pathogen cluster. Indicated are the percentages of positive tested patients for each pathogen. Thus, each colums represent the sum of percentages of the respective cluster. Above the colums, the percentage of positive for *T. whipplei* tested patients of the respective cluster are indicated. The table underneath shows basic characteristics of the cluster as the number of patients per cluster, the mean number of pathogens per sample and the mean age (months).
Abbreviations: n=number, NOP=mean number of positive pathogens/sample

3.4 Longitudinal analysis of *T. whipplei*

3.4.1 T. whipplei longitudinal analysis – Infection/Carriage

Analysing the case and the control group with regards to the longitudinal development of the *T. whipplei* status revealed a significant difference for the higher proportion of children in the control group who, after initially testing negative for *T. whipplei* on V1, kept their negative status throughout both follow-up visits. On the contrary case patients, after testing negative on V1, tended to convert to a positive status at V2 or V3 (p <0,001). In contrast, there was no significant

difference detectable separately for the proportion of children who, after initially testing negative on V1, developed a new *T. whipplei* positive status on V2 and stayed positive on V3 (p=0,245) or returned to their negative status on V3 again (p=1,0). Furthermore, there was no significant difference between the case and the control group in the proportion of initially positive tested children on V1, who lost their positive status of *T. whipplei* on V2 (p=1,0) or on V3 (p=0,245) (Tab. 11).

Comparing the equivalent age subgroups of the control and case group showed that case children from 6–8 months (p=0,013) and 9–12 months (0,024) demonstrated a status conversion from negative on V1 to positive during the time of observation significantly more often than the corresponding control age subgroups.

Besides, there was no significant heterogenity within the case or control age subgroups concerning the longitudinal development of the *T. whipplei* status. However, in the case group, younger children tested positive for *T. whipplei* in all three visits (n=8, two of them 0–5 months, two of them 6–8 months, and four of them 9–12 months); in the control group only children (n=2) older than 9 months presented a positive result for *T. whipplei* on all three visits.

There was no difference between the study groups in the proportion of patients who were tested positive for *T. whipplei* on all three visits (triple positive) (p=0,168) or the proportion of patients who were tested negative for *T. whipplei* on all three visits (triple negative) (p=0,127).

***T. whipplei* control**			**n**
V1+	V2 +	V3 +	2
		V3 -	2
	V2 -	V3 +	7
		V3 -	2
V1 -	V2 +	V3 +	0
		V3 -	0
	V2 -	V3 +	0
		V3 -	69
Total			82

***T. whipplei* case**			**n**
V1 +	V2 +	V3 +	7
		V3 -	0
	V2 -	V3 +	1
		V3 -	2
V1 -	V2 +	V3 +	2
		V3 -	2
	V2 -	V3 +	8
		V3 -	61
Total			83

Table 11 Longitudinal analysis of *T. whipplei*
Reading instructions: The left table represents the control, the right table represents the case group. Start in the first colum on the left for the status on V1 (either positive or negative), then choose the status on V2 (either positive or negative) and in the third colum the status for V3 (either positive or negative). The number (n) in the fourth colum represents the total number of patients with the according longitudinal status in the control (left table) or case (right table) group.

3.4.2 *T. whipplei* longitudinal analysis – clinical presentation

On the date of inclusion case patients who were tested positive on all three visits tended to suffer more often from vomiting compared to case patients with any other *T. whipplei* status (p=0,05) and suffered less often from fever (p=0,04).
In detail, three of the triple *T. whipplei* positive case patients had an exclusive gastrointestinal focus with a clinincal presentation of diarrhoea, vomiting and/or abdominal pain on V1. The other four triple positive tested case patients indicated additionally symptoms of a RTI. There was no alteration of the stool frequency, character, or colour on V1 of triple positive case patients compared to any other status. In the course of the study, three triple positive case patients reported a prolonged episode of acute diarrhoea significantly more often compared to case patients with any other *T. whipplei* status (p=0,02) on V2 – but not compared to triple negative case patients. On V3 there was no specific clinical expression for case patients with a triple positive status detectable. None of these patients suffered from diarrhoea on V3.

In the control group, there was no specific clinical expression detectable for triple positive control patients: Both triple *T. whipplei* positive control patients suffered from fever; one additionally presented symptoms of a RTI on V1. Both patients had no symptoms on V2 and V3.

There was no specific clinical expression on V1 for patients in both study groups with a status conversion from positive on V1 to negative on V2 compared to patients that kept their positive status on V2, including the stool frequency, character, or colour.

On V2, there was no significant clinical symptom for case or control patients who developed a new positive *T. whipplei* status after being tested negative on V1 in general - as well as for patients who developed a new positive status on V2 (after being tested negative on V1) and stayed positive on V3 compared to those who lost their positive status again on V3.
On V3 there was no specific clinical symptom in both study groups for patients who presented a status conversion from negative on V2 to positive on V3.

3.4.3 *T. whipplei* longitudinal analysis – copathogenity and the holistic pathogen profile

Triple positive patients did not show a significant alteration of the NOP on V1 (p_{case}=0,217; $p_{control}$=0,137).
On V2, triple positive children with a history of diarrhoea on V1 presented a higher NOP than case patients with any other longitudinal status trend (p=0,014) and than troughout negative tested case patients (p=0,014) but this observation was no longer detectable on V3 (p=0,42; p=0,559).
In contrast to the case group, the NOP of triple positive control patients neither differed significantly on V2 (p=0,375) nor on V3 (p=0,155) compared to control patients with any other combination of *T. whipplei* results.
Synoptically, there was no significant characteristic of the NOP on any visit in either study group for patients with status conversions from negative to positive across any of the observation points (Tab.17 appendix).

There was no specific pathogen associated with a triple positive status across any of the observation points in both study groups. The above described as significant co-occurrence of Norovirus II on V1 and the co-occurrence of *Campylobacter spp.* on V2 did not show a significant influence on the longitudinal development of the *T. whipplei* status. Neither did the co-occurrence of Norovirus II on V1 support a continued positive result for *T. whipplei* in case from V1 to V2 (OR 0,25 (CI95% 0,02-3,4), p=0,327) nor did a positive result for *Campylobacter spp.* on V1 influence a conversion from negative on V1 to positive on V2 for *T. whipplei* (OR 2,5 (CI95% 0,16-38,6), p=1,0).

There was no association detectable for single pathogens with a triple negative status or a specific status conversion during the time of observation on any of the three study visits in both study groups.
Furthermore, the different longitudinal status combinations occurred equally in the pathogen clusters on V1, V2 and V3.

3.5 Individual and and socio-economical risk factors

The number of people in the household was higher for case patients who were positive for *T. whipplei* on all three visits (mean 15,9 (SD 11,9), p=0,046) compared to triple negative tested patients. The number of people living in one household was not identified as being a statistically significant independent influence on being tested triple negative, converting to a positive status on V2 or V3, or converting to a negative status on V2 or V3 or being tested positive for *T. whipplei* at any point of time.

Considered as a similar indicator of the socio-economic status, the usage of shared bathrooms tended to be more frequent in case patients with a positive result for *T. whipplei* on V1 - though not being statistically significant (p=0,053).

There were no observed significant influences in the case and the control group concerning the habitual contact with animals, supplementation of formula or pap, floor type, house type, immunization status, weight status (normal, under- or overweight) or sex on the point-in-time assessment or the longitudinal development of the *T. whipplei* status on any of the three visit dates.

There was no statistically significant influence of the general prescription of antibiotics as well as for specific agents on the longitudinal development of the *T. whipplei* status on V2 or V3 detectable.

4 Discussion

T. whipplei did not occur more frequently in children with acute diarrhoea compared to controls on the day of inclusion. But an acute diarrhoeal episode might facilitate the infestation with *T. whipplei* within the following month. Beeing tested positive for *T. whipplei* one week after inclusion was associated with a prolonged diarrhoeal episode.

Concomitantly with a positive result for *T. whipplei* a higher average number of other diarrhoeal pathogens was detected for children during the first week after an acute diarrhoeal episode. Norovirus II and *Campylobacter spp.* occurred significantely together with *T. whipplei* in the case group.

Generally, infants in our study population are exposed to diarrhoeal pathogens very early in life. Independently from clinical diarrhoea the prevalence of intestinal pathogens in stool samples of children was high. The average number of pathogens was higher in stool samples from children with diarrhoeal symptoms even 28 days after inclusion.

Focusing on the individual and social risk factors for a *T. whipplei* infection, generally regarded “poor hygienic circumstances” (i.e., a high number of people in a household and the usage of shared bathrooms) could not be proven to be a determinating factor for a *T. whipplei* infection or prolonged carriage at any of the study visits.

4.1 *T. whipplei* prevalence, longitudinal development and clinical expression

To our knowledge, we conducted the first study with a standardized follow-up to assess precisely the persistence of *T. whipplei* and diarrhoeal pathogens during an acute diarrhoeal episode and during the first month after an acute diarrhoea episode of young infants in Africa.

The study site was located within the radius of the second largest city in Ghana and patients from the urban area as well as from rural communities attended equally for medical treatment at the hospital. Comparability of the case and the control group was confirmed by analyzing the essential characteristics of the case and the control group. The detected lower mean body weight percentile is

presumingly due to loss of water due to acute diarrhoea symptoms, though other underlying diseases leading to low body weight cannot be excluded.
The high prevalence of malaria correlates with Ghana as a high-risk area for malaria transmission (Service et al., 2016) but was equally distributed over the case and the control group.

The percentage of *T. whipplei* positive patients without gastrointestinal symptoms in the underlying study for this dissertation resembled the earlier published results of approximately 11% of *T. whipplei* positive healthy children in Africa of approximately the same age (Fenollar et al., 2009, Vinnemeier et al., 2016) but the acquired data do not confirm a higher rate of *T. whipplei* positive children for patients with gastrointestinal symptoms (11,4%) on the day of consultation for medical support (V1) as implied in earlier conducted studies (Raoult et al., 2010, Fenollar et al., 2016, Vinnemeier et al., 2016).

Hitherto acquired numbers of prevalence for *T. whipplei* in African children were gained from cohort studies of two Senegalese villages and contained a heterogenic patient collective with adults and children (Keita et al., 2011) or children from 2 months to 10 years (Fenollar et al., 2009). The cross-sectional study in rural Gabon investigated on humans from 0 to 80 years without specifically regarding the clinical symptoms (Ramharter et al., 2014). The recently conducted case-control study for Ghanainan infants concentrated on children with diarrhoea from 2 months to 15 years but were not adequately powered (Vinnemeier et al., 2016).
In summary, findings for children less than 12 months of age with acute diarrhoea have been rare and for a confirmation of the hypothesis of *T. whipplei* as a diarrhoea-causing agent, a prospective study with a higher number of participants targeting young infants was required.

The prospective study design revealed the obvious difference of a higher percentage of *T. whipplei* positive children with initial gastrointestinal symptoms compared to the control group during the course of the following month - as children with diarrhoeal symptoms tended to stay positive or becoming newly positive for *T. whipplei* whereas the fraction of *T. whipplei* positive children without

gastrointestinal symptoms decreased down to 4,4% before achieving approximately the initial level after one month.
It has to be considered that the diagnosis “diarrhoea/gastroenteritis” has not been defined equally in previous studies and vary from three or more loose stools per day for at least three days (Vinnemeier et al., 2016), to at least three loose stools per day for less than one week (Fenollar et al., 2016) or at least three stools per day (Keita et al., 2011). Another study did not define the diagnosis of diarrhoea at all (Raoult et al., 2010). Our prospective study design allowed a more detailed view of the study collective than study protocols with only one visit that might include children who have been suffering from gastroenteritis for one to e.g. seven days.

Nevertheless, though not being a statistically significant factor on the longitudinal development of the *T. whipplei* status it has to be considered that the entity of prescribed antibiotic agents differed in both study groups. According to the diagnosis related guidelines, Amoxicillin/Sulbactam was prescribed predominately in the control group whereas Metronidazole was prescribed more often in the case group. Amoxicillin has been proclaimed as a medical agent against *T. whipplei* (Boulos et al., 2005), which might as well explain the contrary development of *T. whipplei* occurrence between the case and the control group.

The longitudinal development of the patients’ *T. whipplei* status in the underlying study for this dissertation suggests the hypothesis that diarrhoea facilitates the chronic carriage of – or a reinfection or new infection with *T. whipplei*. In contrast, earlier published data detected a reduction of *T. whipplei* positive children after recovery from gastrointestinal disease (Raoult et al., 2010). But prolonged carriage of *T. whipplei* after a chemical induced damage to the intestinal mucosa has been described in murine models before. The author proclaimed that an impaired mucosal barrier facilitates the invasion of *T. whipplei* leading to an increase of diarrhoea and altered infection blood parameters. Same study could not outrule the hypothesis of *T. whipplei* worsen preexisting mild diarrhoea (Al Moussawi et al., 2011). Congruently, in the TWAS-study a positive PCR result for *T. whipplei* on V2 was the only pathogen associated with a prolonged diarrhoeal episode.

4.2 Diarrhoeal pathogens

Diarrhoeal pathogens were found in children with and without gastrointestinal symptoms although the NOP was significantly higher in the case group throughout the time of observation. On the day of medical consultation, ETEC, Rotavirus and *G. lamblia* were detected signficantly more frequently in the case group and have been found in stool samples from Ghanaian children with diarrhoea before, although out of these three pathogens, only Rotavirus has been described as significantly associated with the development of diarrhoeal symptoms (Eibach et al., 2016, Krumkamp et al., 2015, Ashie et al., 2017).

Although Ghana officially implemented the immunization against Rotavirus in 2012 (Alliance, 2012), there was still a significant difference detectable between the case and control group. It has to be considered that positive PCR-results for Rotavirus can also be caused by vaccination (Gautam et al., 2014), which is scheduled at the 6^{th} and 10^{th} week of life. As in both study groups, the prevalence for Rotavirus was mainly detected before vaccination or months after the second injection; a massive influence of the Rotavirus vaccine on the PCR-results seems unlikely. However, the acquired data show a reduction of Rotavirus associated diarrhoeal episodes as described before (Platts-Mills et al., 2015, Armah et al., 2016, Operario et al., 2017) as only 10,5% of the case group patients were positive for Rotavirus whereas in earlier conducted studies, the percentage for Rotavirus positive Ghanaian children with gastroenteritis was described as approximately 27% (Akuffo et al., 2017, Guandalini et al., 2000, Enweronu-Laryea et al., 2018). Rotavirus as well as ETEC were confirmed as significant pathogens for diarrhoea in young infancy in various study sites all over the world and specifically for the African region as well (Platts-Mills et al., 2015, Kotloff et al., 2013).

In a study from 1990, conducted in rural Ghana, *G. lamblia* was found more often in diarrhoeal stools of children under the age of 5 years before (Nakano et al., 1990). Controversially, recentently released data detected *G. lamblia* equally distributed (Anim-Baidoo et al., 2016, Ashie et al., 2017) or even negatively associated with diarrhoea in Ghanaian children (Eibach et al., 2016).

The in present study observed percentage of *G. lamblia* lies within the range of reported findings in fecal samples from Ghanian children suffering from diarrhoea

which varies broadly from 5,8% to 30,5% (Anim-Baidoo et al., 2016). Children with a *G. lamblia* carriage were mainly observed in the oldest age subgroup of the case group, aligning with earlier reported initial infection rates of children around twelve months of age with gastrointestinal symptoms (Nkrumah and Nguah, 2011, Krumkamp et al., 2015).

Earlier conduced studies detected *Cryptosporidium* as a significant pathogen in children with diarrhoea in Africa and particularly for Ghanaian children under the age of two years (Platts-Mills et al., 2015, Kotloff et al., 2013, Krumkamp et al., 2015). Controversingly, though being detected more frequently in children with diarrhoea, *Cryptosporidia* were not significantly more often present in children with diarrhoea on the day of inclusion.

Also in contrast to earlier acquired data for children in the African region (Operario et al., 2017), as well as explicitly for Ghanaian children of 8-23 months with the identical definition of diarrhoea (Krumkamp et al., 2015), there was no significant influence of Norovirus on the clinical manifestation of diarrhoea detectable in this study.

Hitherto, EHEC has not been identified as a common pathogen in children with and without diarrhoea in developing countries. Compared to figures from Burkina Faso with around 1% positive tested children with diarrhoea, the present observed fraction of approximately 10% EHEC positive children in both study groups on the day of inclusion appears high (Konaté et al., 2017). Multiple studies in developing countries did not identify any child with diarrhoea as EHEC positive at all (Rappelli et al., 2005, Kaddu-Mulindw et al., 2001, Nyanga et al., 2017). Previously in Ghana conducted studies did not imply the detection of EHEC (Ashie et al., 2017, Krumkamp et al., 2015). In the present study, EHEC positive children were recruited throughout the year with an increased presence in October in both study groups. Analysis of the genotypes might clarify whether there might has been a EHEC-outbreak or EHEC is generally more common in the study area than in other developing countries.

4.3 Coinfections

Congruently with earlier conducted studies in Ghana (Eibach et al., 2016) and in developing contries all over the world, multiple different pathogens were observed in samples of the participants of the underlying study with and without diarrhoea. The housing conditions (high number of people sharing one household, high percentage of children living in a house with a soil floor, regular contact to animals) of the participants of the TWAS-study support the assumption of an intensified contact with potential pathogens. In children in developing countries the intestinal colonization have been observed earlier than in children beeing born in a first world country, leading to the assumption that early contact with bacteria supports the colonization with bacteria of the initial sterile gut (Adlerberth et al., 1991, Rotimi et al., 1985).

Congruently with the results of the present study an increasing number of different pathogens per sample with the age during early infancy in developing countries has been observed before. The same studies also observed a higher number of different enteric pathogens for children, living in developing countries with gastrointestinal symptoms compared to children without enteric symptoms. Additional pathogens in young infants' stool samples have been described with increased odds of diarrhoea (Platts-Mills et al., 2015, Kotloff et al., 2013). However, in the underlying data for this dissertation the difference between the case and the control group of the mean number of pathogen per sample remained under the total value of 1 on all three visits. Thus, the impact of this observation remains discussionable.

In this study, particularly children older than 6 months with diarrhoea showed a higher variety of pathogens than the control group on the day of inclusion. Children between 6–8 months of age with diarrhoea carried significantely more pathogens throughout the time of observation compared to the equivalent control group. Infants undergo a complex development of their initially immature immunosystem - beginning with protection through maternal globulines (Marchant et al., 2017, Niewiesk, 2014) and breast feeding until approximately 6 months of age (Moles et al., 2018, Marchant et al., 2017) while expanding their individual immune system due to experiencing more of their personal environment by toddling, walking, or

food supplementation. This expansion of experiences during this vulnerable phase leads to a high number contacts with new enteric bacterias (Palmer et al., 2007, Stark and Lee, 1982) which might be associated with diarrhoea before developing immunity in the following months.

In murine models, an alteration of the microbiome around intestinal wounds that might be caused by inflammation has been observed before (Alam et al., 2016).
But as the reduction of the number of pathogens per sample one week after inclusion and following realignment to approximately the initial level one month after inclusion was observed in children with and without diarrhoea, the potential influence of prescribed antibiotics in the underlying study has to be considered for interpretation, too.

Contradicting earlier results from children of the age between two and four years, in this study a monoinfection with *T. whipplei* was only detected once – in a sample of a child without gastrointestinal symptoms. In fact, the data of the underlying study confirmed earlier observations of a high rate of coinfections with *T. whipplei* (Raoult et al., 2010).

Apart from the higher NOP in children with diarrhoea compared to children without gastrointestinal symptoms, the NOP was significantly higher for children with diarrhoea with a *T. whipplei* positive status (even when excluding *T. whipplei* from this calculation) compared to children with diarrhoea and a negative status for *T. whipplei* on V1 and on V2.
An impaired intestinal microbiome might facilitate the infection with further potential pathogens (Dicks et al., 2017, Stecher, 2015), leading to the hypothesis that the presence and persistence of *T. whipplei* could be a side effect of an impaired gastrointestinal barrier and bacterial mileu during diarrhoeal episodes.

However, according to the acquired data for this dissertation, the quantity of different pathogens per sample assumingly does not independently determine a long time carriage of *T. whipplei*. Children with a throughout positive *T. whipplei* status on all three visits showed a higher NOP only on V2 compared to children with any other *T. whipplei* status. On the contrary, an elevated NOP did not

influence the development of status conversion from negative to positive for *T. whipplei* on both follow-up visits and a low NOP was not associated with a continuous negative result for *T. whipplei.*

Concentrating directly on specified coinfections, some statistically significant co-occurrences with *T. whipplei* were detected at significant levels in children with gastroenteritis: Norovirus II on V1 and and *Campylobacter spp*. on V2. However, neither of these pathogens did show a significant impact on the longitudinal development of the *T. whipplei* status. *Campylobacter spp.* has been found before as a copathogen in stool samples with low bacterial loads of *T. whipplei* of children with diarrhoea (Raoult et al., 2010). The previously reported association of *T. whipplei* with *G. duodenalis* was not confirmed in the underlying study (Fenollar, et al. 2003). But, due to the small number of patients, the actual impact of these co-infections remains unclear.

For the first time, in addition to the investigations of one-on-one co-occurrences of pathogens with *T. whipplei,* a cluster analysis was conducted to determine whether specific pathogen constellations are associated with the occurrence of *T. whipplei.* Regarding the similarity of all positive results of pathogens per patient, case patients with *T. whipplei* were found equally distributed in the clusters in both study groups with one exception: On the first follow up *T. whipplei* was detectable more frequently in a pathogen cluster of case children. Again, *Campylobacter spp.* was found in this cluster in 42,1%. Also Enterovirus was found in 89,5% of the children in the mentioned cluster. But both pathogens also occurred in the other clusters on the respective visit. There is no profound evidence that there are specific pathogen constellations associated with the occurrence of *T. whipplei.* Furthermore in contrast to the further above discussed findings, neither the NOP nor the mean age of that cluster presented extraordinary characteristics.
However, for reliable conclusions further investigations with a higher number of study participants would be required.

4.4 Individual and socio-economic risk factors

Although there was no significant connection detectable in the underlying data, it remains remarkable that children under the age of six months rarely tested positive for *T. whipplei* compared to older age groups.
Noticeably, control patients with a positive *T. whipplei* status on all visits were older than eight months, whereas triple positive case patients occurred in all age subgroups. The hitherto published data confirmed a significantly lower prevalence of *T. whipplei* in healthy children under eight months of age (Fenollar et al. 2009) but the conclusion might be extended that colonization with *T. whipplei* in children under eight months of age might cause gastrointestinal symptoms or that an impairment by diarrhoea of the gastrointestinal system before eight months of age might facilitate a chronic carriage of *T. whipplei*.

Hypothetically, diarrhoea in children older than six months might introduce a carriage of *T. whipplei* as in the underling data a status conversion from negative to positive was significantly more often observed in the case group.
As children loose their maternal immunoprotection at the age between six to eleven months this life period might represent a potential vulnerable phase as the child's individual immune system is developing while intensifying contact with their environment (Fischer Walker et al., 2012). Future studies with a serological analysis of the children's immunostatus might deliver more reliable results.
Breastfeeding has been described as a protecting factor against infectious diseases by alternating the infant's intestinal flora as well as by containing immunological active components (e. g. IgA) (Wold and Adlerberth, 2000). Neither exclusive breastfeeding, nor additional breastfeeding were statistically confirmed as a protecting factor against *T. whipplei* in this study. However, the broad majority in both study groups received breast milk. Therefore the results may not represent the development of children's *T. whipplei* status after weaning from breast milk.
Hirtherto, poor hygienic conditions (Keita et al., 2013b), and close interhuman contact have been proclaimed to facilitate the carriage of *T. whipplei* (Ramharter et al., 2014, Schoniger-Hekele et al., 2007, Fenollar et al., 2008b).
In the underlying data for this dissertation, indicators of clean hygienic conditions such as access to private facilitations were observed more frequently in case patients' families without *T. whipplei* infection though not proven by statistic

significance. As the transmission of *T. whipplei* was described as a human-human transfer, the higher number of people sharing one household of throughout positive tested case patients compared to continuous negative tested patients might underline the intensified interhuman contact as a risk factor for *T. whipplei* carriage. But an increased number of people sharing one household was not confirmed to put one at risk of presenting a *T. whipplei* positive status at any point in time over the course of observation in the present study.

Additionally, there was no significant difference detectable for the said factors for triple positive patients in the control group and no association confirmed for children in both groups between a triple negative *T. whipplei* status and a lower number of people sharing one household. In conclusion, the underlying data support that an intensified inter-human contact and shared bathroom facilities might support a prolonged carriage of *T. whipplei* in children with diarrhoea.

4.5 Limitations

To obtain reliable results the definition for a diarrhoeal episode was strict compared to some other studies. Furthermore, the distance between the study site and the laboratory at the KCCR (Kumasi) and the facility of the Department for Microbiology of the University of Hamburg aggravated the logistic management of the obtained samples and documents. The calculated sample size of 150 children per group could not be obtained. Therefore, the study might be underpowered and the acquired data have to be considered with responsible caution.

Due to the typical pediatric profile of pediatric diseases, control patients were predominantely suffering from RTIs and accordingly treated. Therefore selection and performance bias cannot be excluded.

With regard to the patients' history, specifically concerning the indication of loose stools, we relied on the assessment of the legal guardians. For specification, the questionnaire included the differentiation of watery, mushy, pasty and hard stools as answering possibilities but especially in young infants the specification might be difficult.

There was no stool sample collection of the patients before the onset of the acute diarrhoea episode. Direct interaction of commensal bacteria with the intestinal epithelium can alter the immunoprotective mucosal barrier (Macdonald and

Monteleone, 2005). Furthermore, the anti-inflammatory influence of commensal bacterias via the regulation on immune cells has been observed before (Brestoff and Artis, 2013). Therefore, hypothetically the children of the present study with diarrhoea might have had an impaired intestinal milieu with a weakening impact on anti-inflammatory regulation systems and a concomitant impaired immune protection against enteric pathogens before developing diarrhoea.

Two different methods were used to obtain stool samples. If patients could not provide full stool samples within the defined time windows, a rectal swab was performed to obtain a feces sample. Same swab models were used at the labaratory for the processing of the full stool sample for PCR-assays. Hiertherto, there is no hint of an influence by the direct usage of rectal swabs on the PCR-results instead of taking a controlled amount of stool with the swab from a a full stool sample at the laboratory, though the volume of stool on the swab could not be controlled and the processed sample size might differ.

As there neither a positive control sample nor a reference target were used during the RNA/DNA-purification and analysed during the PCR-analysis, there is no conclusion about the quantitative burden of the individual pathogen and Ct-values (treshold cycle values) were interpretated as a qualitative method indicating whether a particular pathogen was detectable in the respective sample. False negative results due to failure in the RNA/DNA-purification process cannot be excluded. On the contrary, the cut-off for a positive interpretated PCR-result (Ct ≤37) was chosen rather liberally which might have led to some false positive results. But the precondition of two positive DNA-targets (TW27 and TW13) was established to prevent false positive results.

Earlier conducted studies detected high bacterial loads of *T. whipplei* in children with gastrointestinal symptoms compared to asymptomatic or chronic carriers with an active *T. whipplei* replication (Fenollar et al., 2008, Raoult et al., 2010) assuming that symptoms are associated with an active *T. whipplei* replication. Besides improving the understanding of the association of high bacterial loads with *T. whipplei* in young infants and the clinical manifestation of diarrhoea, a quantitative PCR-analysis would clarify if the reduction of the fraction of *T. whipplei* positive children without gastroenteritis on the first follow-up visit might be due to low bacterial loads and the control children were just hovering around the detection level.

4.6 Conclusion

Enteric pathogens occurred regularly in patients with and without gastrointestinal symptoms. The frequency of *T. whipplei* detection in stool samples of Ghanaian children under twelve months of age was in line with earlier findings in Ghanaian and Senegalese populations. But as *T. whipplei* was not found significantly more often in children with diarrhoea on the day of medical consultation the hypothesis of *T. whipplei* as a diarrhoea-causing agent in young infants could not be supported with these data.

The persistant occurrence of *T. whipplei* in children with gastrointestinal symptoms during the follow-up period in combination with the observed increased number of pathogens suggests an alternative hypothesis: that an impaired intestinal mucosa or bacterial mileu after a diarrhoea episode might facilitate the colonization and new infections with miscellaneous enteral pathogens including *T. whipplei* and might be associated with the clinical expression of prolonged diarrhoea.

Particularly the phase after the sixths months of age might represent a particular vulnerable phase defined by the gastrointestinal colonization of variable pathogens, including *T. whipplei*.

Defined pathogens as Norovirus II and *Campylobacter spp.* might be associated with the occurrence of *T. whipplei* and potentially be associated with the clinical manifestation of diarrhoea.

For a better understanding, studies needs to be designed that investigate on the origin for the multiple pathogen carriages in children with and without diarrhoea and the clinical impact on the expression of gastrointestinal symptoms.

Furthermore, studies to investigate the colonization process with potential pathogens including *T. whipplei* after a diarrhoeal episode and the impact on the duration of diarrhoea episodes are required.

In future, case-control studies with longitudinal design including a the collection of stool samples before developing diarrhoea, a quantitative PCR-method, serological analysis and a higher number of study patients might create a more differentiated understanding on the clinical impact of new infections and chronic colonization with *T. whipplei.*

5 Abstract

For decades, *T. whipplei* has been associated with causing the rare tropical Whipple's disease in white men. However, recent data describe the higher prevalence of *T. whipplei* a) in humans working and living under poor hygienic circumstances and b) in children with diarrhoea. Subsequent studies confirmed a higher prevalence of *T. whipplei* in children, especially young infants with diarrhoea in countries with low hygienic standards. This study investigated the correlation of the expression of gastrointestinal symptoms in parallel with the occurrence of *T. whipplei* in stool samples of children under twelve months of age, including two follow-up visits during a 28 days period. Furthermore, individual and socio-economic risk factors as well as coinfections with potentially enteric pathogens were evaluated by questionnaires and PCR-analysis. The prevalence of *T. whipplei* in individuals without gastrointestinal symptoms – 13,1% – resembled the earlier published results of healthy children in Africa. However, in contrast to earlier findings, the prevalence of *T. whipplei* in stool samples was not associated with the manifestation of diarrhoea on the day of medical consultation. Follow-up visits unmasked the relatively higher prevalence of *T. whipplei* in stool samples of children with an initial history of diarrhoea on day six after the first visit at the outpatients department. Interestingly, the statistically significant difference of the prevalence is in part due to the decrease of the percentage of *T. whipplei* positive patients in the control group. Specific pathogens such as Norovirus Genotype II and *Campylobacter spp.* might be particularly associated with the occurrence of *T. whipplei* in children with diarrhoea. A general impairment of the gastrointestinal bacterial mileu might influence a chronic carriage of *T. whipplei.* The impact of hygienic cofactors could not be confirmed clearly.

In summary, diarrhoea might facilitate the colonization with miscellaneous enteric pathogens, including *T. whipplei* and might be associated with the clinical expression of prolonged diarrhoea. However, for confirmation of the reported findings, further longitudinal studies in healthy children experiencing acute diarrhoea while being followed up allowing the inclusion of pre-diarrhoeal samples as a baseline status with a higher number of study patients are required.

Zusammenfassung

Jahrzehntelang wurde *T. whipplei* vorwiegend als Erreger des Morbus Whipple betrachtet - eine seltene Tropenkrankheit weißer Männer. Jedoch zeigen aktuellere Daten eine höhere Prävalenz von *T. whipplei* bei a) Menschen, welche unter schlechten hygienischen Bedingungen leben und arbeiten b) bei Kindern mit Diarrhoe. Folgestudien bestätigten eine höhere Prävalenz bei Kindern, insbesondere bei kleinen Kindern mit Diarrhoe in Entwicklungsländern. Die zugrundelegende Studie untersuchte den Zusammenhang zwischen gastrointestinalen Symptomen und dem Vorkommen von *T. whipplei* im Stuhl bei Kindern im Alter von unter 12 Monaten und beinhaltete zwei Folgebesuche innerhalb von 28 Tagen. Zusätzlich wurden individuelle und sozio-ökonomische Risikofaktoren anhand von Fragebögen erfasst, sowie Koinfektionen mittels PCR-Analyse detektiert. Individuen ohne gastrointestinale Symptome wiesen mit 13,1% eine vergleichbare Prävalenz von *T. whipplei* zu früher veröffentlichen Daten gesunder afrikanischer Kinder auf. Im Gegensatz zu zuvor veröffentlichen Daten zeigten Patienten mit klinisch apparenter Diarrhoe keine erhöhte Prävalenz von *T. whipplei* am Tag der medizinischen Konsultation. Folgebesuche demaskierten eine Verschiebung der Relation *T. whipplei* positiver Patienten an Tag 6 nach Inklusion - mit einer verhältnismäßig erhöhten Prävalenz bei Kindern, welche initial bei Studieneinschluss an Diarrhoe gelitten hatten. Interessanterweise resultiert der statistisch signifikante Unterschied der Prävalenz vorwiegend aus der Dezimierung des *T. whipplei* positive getesteten Patientenanteils der Kontrollgruppe. Einzelne Erreger, wie Norovirus Genotype II und *Campylobacter spp.* sind potenziell mit dem Auftreten von *T. whipplei* bei Kindern mit Diarrhoe assoziiert. Eine Veränderung des gastrointestinalen Bakterienmilieus hat wohlmöglich Einfluss auf die Dauer einer Besiedelung mit *T. whipplei.* Der Einfluss hygienischer Kofaktoren konnte nicht eindeutig bestätigt werden.

Zusammenfassend könnte eine diarrhoeische Episode die gastrointestinale Besiedelung durch Darmpathogene, inklusive *T. whipplei* begünstigen und mit einer verlängerten klinischen Manifestation von Diarrhoe vergesellschaftet sein. Um die beobachteten Ergebnisse zu bestätigen sind weitere longitudinal angelegte Studien mit einer Probenkonservierung im gesunden Intervall vor einer diarrhoeischen Episode mit einer höheren Patientenzahl erforderlich.

6 List of Abbreviations

AF	Attributable Fraction
C	Celcius
C.	Campylobacter
ddH_2O	Double-distilled water
DNA	Desoxyribonucleic acid
E. dispar	Entamoeba dispar
EAEC	Enteroaggregative *Escherichia coli*
EIEC	Enteroinvasive *Escherichia coli*
EPEC	Enteropathogenic *Escherichia coli*
ETEC	Enterotoxic *Escherichia coli*
G. lamblia	Giardia lamblia
GIT	Gastrointestinal tract
h	hours
HIV	Human Immunodeficiency Virus
KCCR	Kumasi Center for Colloborative Research
km	kilometer
KNUST	Kwame Nkrumah University of Science and Technology
µg	microgram
m	months
ml	milliliter
n	number
NOP	Number of pathogens per sample
Norovirus II	Norovirus genotype II
OPD	Outpatient department
OR	Odds ratio
PCR	Polymerase chain reaction
PK	Protein kinase K
pmol	picomol
RNA	Ribonucleic acid
RTI	Respiratory tract infection
SD	Standard Deviation
spp.	Species
ST-ETEC	Heat-stable enterotoxin *Escherichia coli*
T. whipplei	Tropheryma whipplei
TW	Tropheryma whipplei
TWAS	Tropheryma whipplei in Africa study
V1	Visit one, day of medical consultation at the OPD and inclusion
V2	Visit two, follow-up visit on day six after inclusion
V3	Visit three, follow-up visit on day 28 after inclusion
WD	Whipple's disease
WHO	World Health Organization
95%CI	95% confidence intervall

7 Appendix

	Rotavirus/ Norovirus1/ Norovirus2	**Adenovirus/ Astrovirus**	**Sapovirus/ Enterovirus**	**EHEC/EPEC/ Salmonella/ Shigella/EIEC**
5x Master Mix (µl)	5	5	5	5
H2O (µl)	10,25	11,75	6,75	3,5
GlobalMix (µl) (Primer/Sonden)	4,5	3	3	9
Enzym (µl)	0,25	0,25	0,25	0
IC Assay (µl)	0	0	2,5	2,5
Control RNA (µl)	0	0	2,5	0
Total amount (µl)	20	20	20	20

Table 12 Pipetting Scheme PCR I

	ETEC/EAEC	**Campylobacter**	**Protozoa**	**Tropheryma whipplei**
5x Master Mix (µl)	5	5	5	5
H2O (µl)	6	12	10,5	8,5
GlobalMix (µl) (Primer/Sonden)	9	3	4,5	1,5
Enzym (µl)	0	0	0	
IC Assay (µl)	0	0	0	2,5
Control RNA (µl)	0	0	0	2,5
Total amount (µl)	20	20	20	20

Table 13 Pipetting Scheme PCR II

V2		Control (n=90)		Case (n=96)		p
		n	%	n	%	
Sex	Male	52	57,8	44	51,0	0,109
	Female	38	42,2	52	54,2	
Age (months)	Mean (SD)	6 (3)		7 (3)		0,562
	0-5	32	35,6	31	32,3	0,852
	6-8	34	37,8	36	37,5	
	9-12	24	26,7	29	30,2	
Weight (percentile)[a]	Mean (SD)	34 (28)		29 (29)		0,07
	Under 3.	10	11,1	14	14,6	0,739
	3.-97.	75	83,3	76	79,2	
	Over 97.	2	2,2	3	3,1	
Nutrition[b]	Exclusive breast feeding	7	7,8	5	5,2	0,865
	Breast feeding and formula supplementation	76	84,4	84	87,5	
	Only formula supplementation	1	1,1	2	2,1	
Immunization[c]	Up to date	75	83,3	79	82,3	0,621
People in household	Mean (SD)	8 (6)		10 (12)		0,966
Contact to animals	Yes	53	58,9	59	61,5	0,765
	No	37	41,1	37	38,5	
House[d]	Stone, cement, brick house	76	84,4	79	82,3	1,0
	Mud house	13	14,4	13	13,5	
	wood house	1	1,1	3	3,1	
Floor[e]	Cement floor	39	43,3	32	33,3	0,106
	Mud house	13	52,2	13	55,2	
	Wood house	1	4,4	3	8,3	
Bathroom[f]	Own bathroom of the family	30	33,3	35	36,5	0,759
	Shared bathroom	59	65,6	61	63,5	

Table 14 V2 Individual and socio-economic patients' characteristics
Missing values [a]=3 controls, 3 cases, [b]=2 controls, 1 case, [c]=14 controls, 14 cases, [d]=1 case, [e]= 3 controls, [f]=1control, 3 cases

V3		Control (n=87)		Case (n=89)		
		n	%	n	%	p
Sex	Male	50	57,5	40	44,9	0,101
	Female	37	42,5	49	55,1	
Age (months)	Mean (SD)	7 (3)		7 (3)		0,791
	0-5	29	33,3	28	31,5	0,739
	6-8	33	37,9	32	36,0	
	9-12	25	28,7	29	32,6	
Weight (percentile)[a]	Mean (SD)	36 (29)		30 (30)		0,07
	Under 3.	9	10,3	11	12,4	0,739
	3.-97.	72	82,8	72	81,0	
	Over 97.	3	3,4	3	3,4	
Nutrition[b]	Exclusive breast feeding	7	8,0	6	6,7	0,977
	Breast feeding and formula supplementation	74	85,1	76	85,4	
	Only formula supplementation	1	1,5	2	2,2	
	Other	4	5,6	4	4,5	
Immunization[c]	Up to date	73	83,9	73	82,0	062
People in household	Mean (SD)	8 (6)		7 (3)		0,649
Contact to animals	Yes	53	60,9	56	62,9	0,877
	No	34	39,1	33	37,1	
House[d]	Stone, cement, brick house	72	82,8	70	78,7	0,897
	Mud house	13	14,9	15	16,9	
	Wood house	2	2,3	3	3,4	
Floor[e]	Cement floor	37	42,5	32	36,0	0,449
	Soil floor	46	52,9	46	51,7	
	Other floor	4	5,6	6	6,7	
Bathroom	Own bathroom of the family	28	32,2	30	33,7	1,0
	Shared bathroom	58	66,7	59	66,3	

Table 15 V3 Individual and socio-economic patients' characteristics
Missing valus [a]=3 controls, 3 cases, [b]= 1 control, 1 case, [c]=13 controls, 13 cases,
[d]=1 case, [e]=1 control, 3 cases

V1, V2 and V3		Control (n=82)		Case (n=83)		
		n	%	n	%	p
Sex	Male	47	57,3	38	45,8	0,162
	Female	35	42,7	45	54,2	
Age (months)	mean (SD)	6 (3)		7 (3)		0,631
	0-5	29	35,4	26	31,3	0,819
	6-8	31	37,8	32	38,6	
	9-12	22	26,8	25	30,1	
Weight (percentile)[a]	Mean (SD)	35 (28)		30 (31)		0,084
	Under 3.	9	11,0	11	13,3	0,826
	3.-97.	68	82,9	66	79,5	
	Over 97.	2	2,4	3	3,6	
Nutrition[b]	Exclusive breast feeding	7	8,5	5	6,0	0,929
	Breast feeding and formula supplementation	69	84,1	71	85,5	
	Only formula supplementation	1	1,2	2	2,4	
	Other	4	4,9	4	4,8	
Immunization[c]	Up to date	69	84,1	71	85,5	0,62
People in household	mean (SD)	8 (6)		10 (12)		0,656
Contact to animals	Yes	48	58,5	51	61,4	0,752
	No	34	41,5	32	38,6	
House[d]	Stone, cement, brick house	68	82,9	67	80,7	0,749
	Mud house	13	15,9	13	15,7	
	Wood house	1	1,2	3	3,6	
Floor[e]	Cement floor	36	43,9	29	34,9	0,221
	Soil floor	42	51,2	43	51,8	
	Other floor	4	4,9	7	8,4	
Bathroom[f]	Own bathroom of the family	27	32,9	28	33,7	1,0
	Shared bathroom	54	65,9	55	66,3	

Table 16 V1, V2 and V3 Individual and socio-economic patients' characteristics
Missing valus [a]=3 controls, 3 cases, [b]= 1 control, 1 case, [c]=12 controls, 9 cases, [d]=1 case, [e]= 3 cases, [f]=1 control

NOP	Control			Case		
	Triple positive (n=2)	**Any** (n=80)	**p**	**Triple positive** (n=7)	**Any** (n=76)	**p**
V1	4,0 (1,4)	2,2 (1,6)	0,137	3,6 (1,0)	2,9 (1,8)	0,217
V2	3,0 (2,8)	1,8 (1,4)	0,375	3,7 (1,0)	2,3 (1,7)	0,014
V3	4,0 (1,4)	2,3 (1,5)	0,155	3,9 (2,1)	3,0 (1,6)	0,42
	Triple negative (n=69)	**Triple positive** (n=2)	**p**	**Triple negative** (n=61)	**Triple positive** (n=7)	**p**
V1	2,2 (1,6)	4,0 (1,4)	0,095	2,8 (1,7)	3,6 (1,0)	0,136
V2	1,8 (1,3)	3,0 (2,8)	0,399	2,2 (1,7)	3,7 (1,0)	0,014
V3	2,3 (1,6)	4,0 (1,4)	0,133	3,2 (1,6)	3,9 (2,1)	0,559
	New positive V2 (n=0)	**V1 and V2 negative** (n=69)	**p**	**New positive V2** (n=4)	**V1 and V2 negative** (n=69)	**p**
V1	–	2,17 (1,6)	–	3,0 (1,4)	3,0 (1,4)	0,457
V2	–	1,8 (1,3)	–	2,5 (1,9)	2,5 (1,9)	0,87
V3	–	2,3(1,6)	–	2,0 (0,8)	2,0 (0,8)	0,072
	New positive V3 (n=0)	**Triple negative** (n=69)	**p**	**New positive V3** (n=8)	**Triple negative** (n=61)	**p**
V1	–	2,2 (1,6)	–	2,8 (2,1)	2,8 (1,7)	0,856
V2	–	1,8 (1,3)	–	2,3 (1,9)	2,2 (1,7)	0,924
V3	–	2,3 (1,6)	–	2,4 (1,7)	3,2 (1,6)	0,253

Table 17 NOP of various longitudinal *T. whipplei* status
Indicated are the different pathogens per sample (NOP) without counting *T. whipplei.*
Abbreviations: Tripple positive: positive for *T. whipplei* on all visits; Triple negative: negative for *T. whipplei* on all visits; New positive V2: negative on V1, positive on V2; New positive V3: negative on V1 and V2, positive on V3

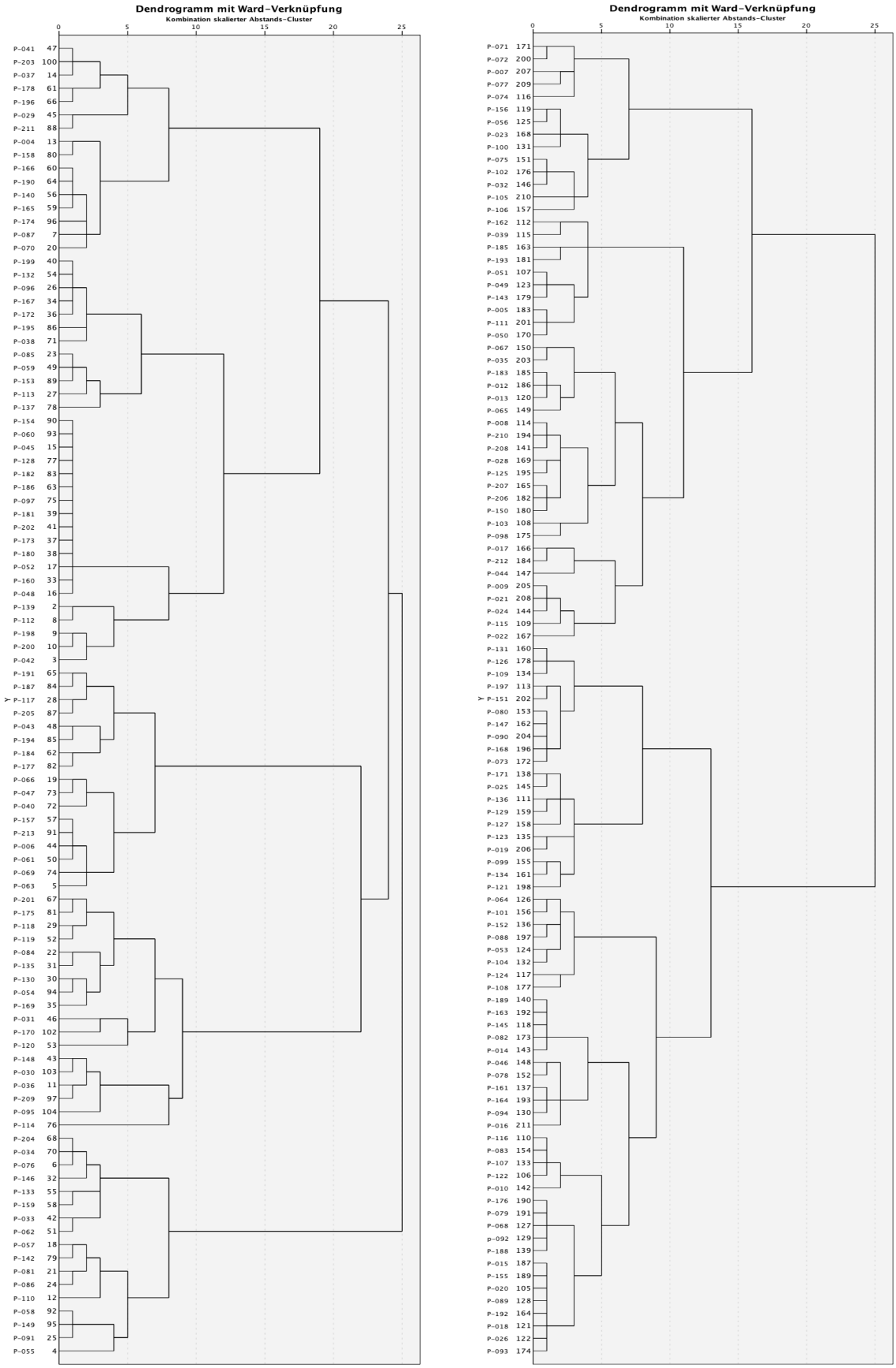


Figure 13 V1 Dendrogramm according to similarity of the pathogen profile of the control group (left) and the case group (right)

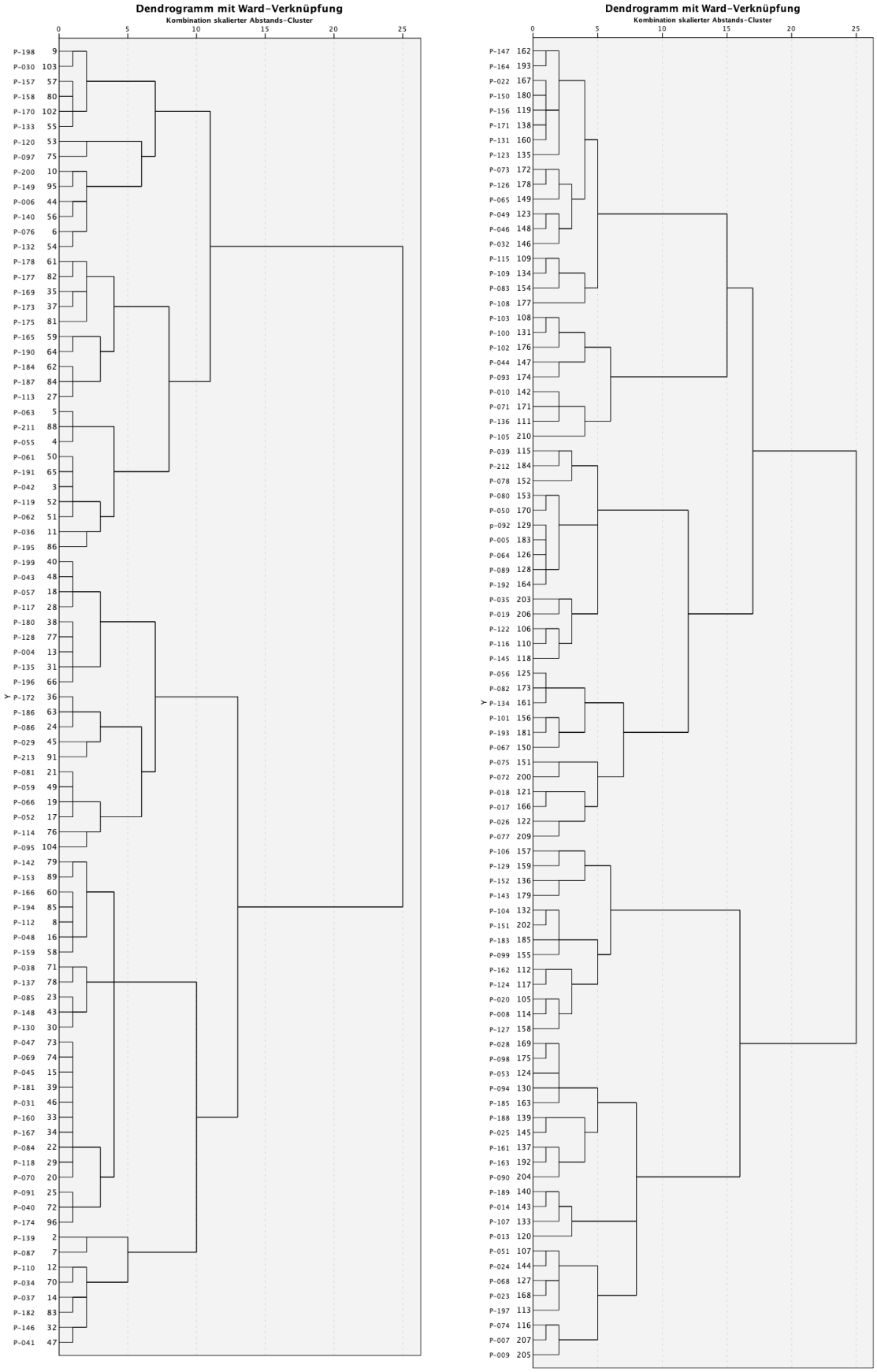


Figure 14 V2 Dendrogramm according to similarity of the pathogen profile of the control group (left) and the case group (right)

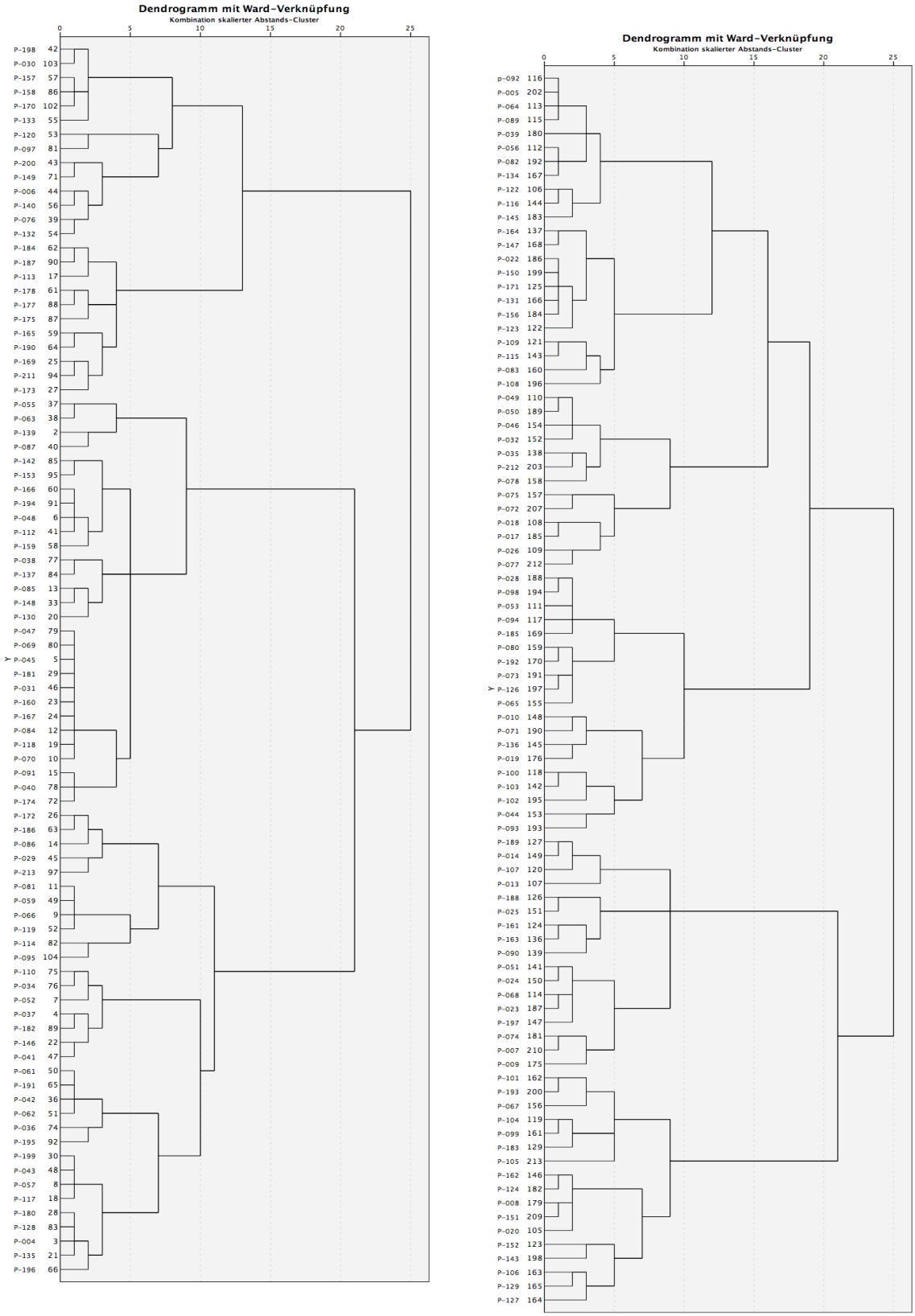


Figure 15 V3 Dendrogramm according to similarity of the pathogen profile of the control group (left) and the case group (right)

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9 LEBENSLAUF

Lebenslauf aus datenschutzrechtlichen Gründen nicht enthalten

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