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Epidemiological investigations on the public health significance of C*ryptosporidium* parasites in livestock and people in the Ismailia Canal Zone of Egypt

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To my home country, Egypt

To my beloved family

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Abbreviations

ETEC	Enterotoxic *Escherichia coli*
C.	*Cryptosporidium*
CDC	Center for Disease Control
18S rRNA	18S ribosomal ribonucleic acid
COWP	*Cryptosporidium* oocyst wall protein
ABC	ATP-binding cassette
KDa	70 kilo Dalton
HSP70	Heat shock protein
GP60	60-kDa glycoproteins
PCR	Polymerase chain reaction
CDPKs	Calcium-dependent protein kinases
DVG	Deutsche Veterinärmedizinische Gesellschaft
SCID	Severe combined immune deficiencies
UV	Ultraviolet
FDA	Food and Drug Administration
IFA	Immunofluorescent antibody-based
ELISA	Enzyme linked immunosorbent assays
EITB	Enzyme-linked immunoelectrotransfer blots
EIA	Enzyme immunoassay
RAPD-PCR	Random amplified polymorphic DNA PCR
AP-PCR	Arbitrary primed PCR
RT-PCR	Reverse transcription PCR
RFLP	Restriction fragment length polymorphism
SSCP	Single strand conformation polymorphism
BLAST	Basic Local Alignment Search Tool
FMD	Foot and Mouth Disease
LSD	Lumpy Skin Disease

1 Introduction

The protozoon *Cryptosporidium* is a worldwide cause of enteric infections in both humans and animals, and, due to the zoonotic character of some of its species, is among the most relevant parasitic enteric agents in human and veterinary medicine.

The cause of the cryptosporidiosis named enteric disease is highly dependent on the immune status of the host. While in immunocompetent individuals *Cryptosporidium* infections most commonly result in acute self-limiting gastroenteritis, cryptosporidiosis in immunocompromised individuals can develop into a chronic and life-threatening diarrheal disease. Due to their immature immune status, neonates are highly susceptible for infections with *Cryptosporidium* and routinely get infected by oral uptake of even low infective doses of the parasite's oocysts. The oocysts can survive for several months and retain infectivity in a latent form outside the host, despite adverse environmental factors, including salinity and chemicals (Fayer et al., 1998b; Sunnotel et al., 2006; Smith et al., 2007). The intestinal malabsorption caused by cryptosporidiosis in developing countries further affects particularly malnourished children (Desai et al., 2012). It was estimated that 1 to 10% of the populations in developing countries were infected with *Cryptosporidium*, wherein 1-to-9-year-old children and toddlers were the most affected groups (Chen et al., 2003).

In animals, once considered to cause opportunistic infection, *Cryptosporidium* now, together with enterotoxic *Escherichia coli* (ETEC) and Rota- and Corona-viruses, is considered an important component of the calf diarrhea complex (Gulliksen et al., 2009).
In human and veterinary medicine, Nitazoxanide and Halofuginone are approved drugs for treatment, respectively pro- and metaphylaxis; their application in all cases of human and animal cryptosporidiosis though does not guarantee an effective treatment. To date, no unrestrictedly effective chemotherapeutics do exist for the treatment of cryptosporidiosis (Stockdale et al., 2008; Shahiduzzaman and Daugschies, 2012). Consequently, control of cryptosporidiosis has to rely on reducing the prevalence of the parasite and on breaking the transmission pathways of *Cryptosporidium* species causing disease in animals, transmitting them to humans (zoonotic) or those perpetuating infection in humans only (anthroponotic). To achieve effective control, information on the magnitude of infections, the spatial distribution of species and in risk groups of animals and on the major sub-species prevailing in animals and humans is essential. Such epidemiological information to initiate planning of control measures is limited or totally absent in many countries.

In Egypt, reports describing *Cryptosporidia* and cryptosporidiosis are few and scanty. In particular, a systematic evaluation of the current situation is missing. There is a paucity of information from representative field studies on cryptosporidiosis both in livestock and humans, particularly from zones of the country where people and livestock live in close density. As more than 80% of Egypt is desert and has no effective rainfall, the Nile River and systems of irrigation canals are the main water sources. The Ismailia Canal Zone is such an area, where sizeable human populations and cattle and buffaloes are concentrated along and share the narrow banks of the Suez Canal and a system of lateral canals. Smallholder farmers not owning agricultural lands are the major livestock producers. Husbandry is poor and stock and drinking water are inadequate and/or of poor quality. Little systematic knowledge about the epidemiology and genetic characteristics of *Cryptosporidium* spp. in the animals and humans exists in the region.

This study therefore did choose an epidemiological investigation to provide a first approximately representative overview on the importance of cryptosporidiosis in Ismailia province.

This dissertation aims to:

1- provide informations on the frequencies, distributions, risk factors and infection dynamics of *Cryptosporidium* in and among cattle/buffaloes and diarrheal children in the Ismailia province by way of an epidemiological (designed) study.

2- give an overview of the genetic diversity, population structures and zoonotic or anthroponotic genotypes of *Cryptosporidium* and their transmission pathways in and between respective livestock and children in the study area.

The two publications in this thesis addressing above objectives were, for technical reasons, finalized and published inversely, the embracing molecular analysis in publication (1) first, in 2013, the baseline epidemiological publication (2) second, in 2014. For logical reasons, in this thesis publication 2 is followed by publication 1.

2 Review of literature

2.1 The *Cryptosporidium* parasite

2.1.1 Brief history of the *Cryptosporidium* agent

Discovery and naming of the genus *Cryptosporidium* (*C.*) go back to Ernest Edward Tyzzer (1875-1965). In his research Tyzzer could repeatedly detect a parasitic agent in the stomach glands of domestic mice (*Mus musculus*) and already in 1907 described its sexual and asexual development stages (sporozoites, schizonts, microgamont, microgametes, macrogamont, spore (oocyst)) as well as its particularity in the form of an "organ of attachment". Tyzzer already then raised the possibility of a monoxious development cycle and a faecal-oral transmission pathway. Due to the absence of an assured taxonomic status, Tyzzer for his discovery chose the name *Cryptosporidium muris* (Tyzzer, 1907).

In 1910, Tyzzer wrote a detailed description of the agent, which further included a wider host spectrum and the hypothesis of a possible auto-infection. He proposed the generation of a genus *Cryptosporidium* (*C.*) (crypticus, latin for hidden; here concealed sporocyst) with the respective species *C. muris* (Tyzzer, 1910). Except for the fact that Tyzzer described the localization of the development stages as extra-cellular, all his microscopic observations on the development cycle of *C. muris* could be confirmed electron-microscopically (Fayer et al., 1990a).

Two years later, Tyzzer described another new species, *C. parvum*, which he also detected in house mice. This species differs from *C. muris* morphologically (smaller oocysts) as well as in its predilection site (the small intestine' epithelial layer) (Tyzzer, 1912).

In the following years, no further studies to research this parasite were carried out, as it was considered to have neither economic nor (veterinary) medical importance. In 1955 a new species, *C. meleagridis*, indeed was associated with diarrhea and deaths in turkey chicks (Slavin, 1955), but *Cryptosporidia* essentially entered veterinary medicine only in the early 1980s with reports of *Cryptosporidium*-associated calf diarrhea (Meuten et al., 1974). Only in the 1990s, Tzipori et al. (1983) and Heine et al. (1984) established that *Cryptosporidium* is a primary entero-pathogen, able to cause neonatal calf diarrhea.

In 1976 the first two case reports for human cryptosporidiosis were published (Meisel et al., 1976; Nime et al., 1976). Human medical relevance of *Cryptosporidium* started in 1982, with a report of the US "Center for Disease Control" (CDC) on *Cryptosporidium*-induced diarrheas in HIV/AIDS patients. The globalized interest and importance of *Cryptosporidium* as a public health problem began in 1993, when more than 400,000 residents in Milwaukee, Wisconsin, USA were affected by *C. hominis* due to the

consumption of contaminated drinking water, to be marked as the world's largest recorded waterborne outbreak (MacKenzie et al., 1994; Peng et al., 1997; Thompson et al., 2008).

2.1.2 Taxonomy

To date, the taxonomic status of the genus *Cryptosporidium* remains enigmatic, new molecular discoveries on the speciation of the genus continue to be a challenge to taxonomists. Traditionally, *Cryptosporidia* as protozoan parasites due to great similarities are classified in the Coccidia class of the phylum Apicomplex, although *Cryptosporidia* show features which differ them from all other Coccidia (Hijjawi et al., 2002): among them are the intracellular, but extracytoplasmic localization, the forming of a "feeder" organ, the forming of two both morphological (thin- or thick- walled) and functional (auto versus new-infection) types of oocysts, the small size of oocysts, missing morphological characteristics like e.g. sporocysts or micropyles and the insensitivity of *Cryptosporidia* to all so far tested anti-coccidial compounds (Hijjawi et al., 2002; Smith and Corcoran, 2004).

A phylogenetic study of Carreno et al. (1999), based upon a comparison of "small-subunit ribosomal RNA" gene sequences, permits to conclude a closer affinity of *Cryptosporidia* with the gregarines (Apicomplexa: Gregarinasina). This hypothesis is supported by studies of Hijjawi et al. (2002) and Rosales et al. (2005) in which they observed "gregarine-like" development stages of *Cryptosporidia* during *in vitro* agent cultivation. Further indicators for a possible relatedness to the gregarines are in details described in a publication of Karanis and Aldeyarbi (2011).

The lack of distinctive morphological characteristics of the only exogenous stage, the oocyst (only slight variations in size), for a long time did not permit a definite species classification. Characteristics such as the morphology of endogenous and exogenous development stages, the predilection site and the host specificity served for species identification and naming of the Apicomplexa (Fayer, 2010).

Only with the involvement of molecular-biological methods, features and differences in gene sequences like the base sequence of the 18S ribosomal "ribonucleic acid" (rRNA, of the acetyl-CoA-synthesis gene) or the "*Cryptosporidium* oocyst wall protein" (COWP) can be used to identify species-specific characteristics and thus enable a definite species identification (Morgan et al., 1998; Xiao, 2010).

Molecular-biological methods also led to the realization that the two different infection pathways of human cryptosporidiosis (zoonotic and anthroponotic) are due to two

genotypes of *C. parvum*. The genotype responsible for human-to-human transmission was named genotype I or "human genotype", whereas the genotype transmitted by animal contact was named genotype II or "bovine genotype". Genotype I later was defined as an own species and re-named into *C. hominis*. Genotype II is still perpetuated as *C. parvum* (Morgan-Ryan et al., 2002).

2.1.3 Development cycle and morphology

Cryptosporidia are parasitizing protozoa with a complex monoxenous development cycle (life cycle) which can be divided into an asexual (sporogony and schizogony/merogony) and a sexual (gamogony) phase. During the invasion of the free living stages, proliferation and differentiation take place within a unique parasitophorous vacuole under the host cell brush border, but outside the host cell cytoplasm (Leitch and He, 2012). Like Coccidia, *Cryptosporidium* thus attaches to the cell surface and undergoes gliding mobility, a process in which the parasites move along the cell surface for a short time, before they start to enter the cell. However, unlike Coccidia, they do not invade the cell actively, but rather trigger the cell to embrace them with a host cell-derived membrane. As a result, *Cryptosporidia* do not fully invade the cell, but rather stay in an epicellular location. At the parasite-cell interface, *Cryptosporidium* forms an actin-rich disk, a feeder organelle that is thought to be responsible for nutrition intake, and a small channel funneling into the host cell cytoplasm (Lendner and Daugschies, 2014).

The infection-inducing agent of *Cryptosporidium* is the sporulated thick-walled oocyst which can survive for a long time outside the host and is resistant to many disinfectants such as chlorine (Fayer and Xiao, 2007). The on average 4µmx6µm large *Cryptosporidium* oocysts (Table 1) are of spheric to ovoid shape and contain, further to a residual body, 4 banana like or comma-shaped sporozoites with a pointed front end and a stubbed hind end, in which's rear one-third the nucleus is localized (Upton and Current, 1985; Current and Reese, 1986; Fayer and Ungar, 1986). The 2,4µmx2,5µm large residual body consists of a spherical to ovoid membrane-bound globule (about 1,5µmx1,6µm), surrounded by several small granules (about 0,2µmx1,2µm). The alongside and parallel aligned sporozoites are closely attached.

Characteristic for the genus *Cryptosporidium* is that the sporozoites are not additionally encapsulated by a sporocyst. The smooth colorless oocyst wall consists of two layers (an outer and an inner layer) and a pre-formed junction does extend as a pale line from one

pole partially above the circumference of the oocyst, reaching or, respectively, running from one oocyst pole diagonal up to one third (maximum half) of the oocyst' circumference. The about 5µmx1µm large four sporozoites hatch out of this pre-formed joint in the gastrointestinal tract under the influence of changes in temperature and pH, CO_2, gall bladder salts and pancreas enzymes. The last three factors though do not appear necessary for excystation, so that also an extra-intestinal colonization of e.g. the respiratory tract is possible. The free sporozoites with their proximal end adhere to the microvilli seam of the enterocytes and by this activate their internalization. For *C. parvum*, for human ileo-caecal adeno-carcinoma cells (HCT-8 cells) and primary ileal enterocytes of neonatal mice (age of 5 days), an involvement of the sporozoites' surface protein 47 in the function of a ligand was demonstrated in this process.

The respective host cell receptor is a 57 kDa protein (p57). The 40 kDa and >900 kDa sporozoites' glycoproteins (GP40, GP900) and the "circumsporozoite-like" (CSL) glycoprotein also seem to take part in the adhesion and invasion process (Smith et al., 2005; O'Hara and Chen, 2011). The host cell is triggered to embrace the sporozoites with membrane protrusions, thereby forming a parasitophore vacuole within the brush border of the enterocyte; a localization of the parasitophore vacuole different from that of other Apicomplexa, and frequently described as intracellular, but extracytoplasmic (O'Hara et al., 2004).

In the cause of internalization at the contact point between sporozoit and host cell-membrane, a unique multiple/extensive folded membrane structure, the so-called "feeder" organelle develops. The supply with nutrients of the maturing parasite is likely facilitated by this organelle (Zapata et al., 2002). In a localization study, the organelle was able to demonstrate aggregations of the "ATP-binding cassette" (ABC) protein CpABC1 (*C. parvum* ABC protein) in a hemispheric structure at the border area between host cell and mature meront. As most ABC proteins are ATP-dependent membrane transporters, it can be hypothesized that CpABC1 in this function takes part in metabolic activities between host cell and maturing parasite. Following the internalization process, the sporozoit within the parasitophore vacuole differentiates to a about 4µmx4µm large spherical trophozoite with an excentric positioned cell nucleus. By three asexual divisions (merogony/schizogony), it is developed to the about 5µmx5µm large type-1 meront, which contains, as a consequence of a-synchronous cell nucleus division, up to 8 merozoites. The position of the cell nucleus of these motile stages, compared to sporozoites, is more

central. Otherwise, the merozoites resemble the sporozoites in shape and size and also join some epitope with these.

After leaving the parasitophore vacuole, the merozoites either initiate a further asexual cycle; analog to the sporozoites, they infest further epithelial cells and again develop to type-1 meronts. Alternatively, they initiate the sexual development phase by passing through a stage conversion to the slightly smaller type-2 meront. Within this meront type, 4 merozoites develop by asexual division, which, after infestation of further enterocytes, differentiate into micro- and macro-gametes (gamogony). The spheric, 5µmx4,5µm large immature micro-gamontes contain up to 16 peripherally located compact cell nuclei, precursors of the developing micro-gametes. These are of bullet shape with the stubbed front end, are non-flagellated and nearly are filled out by their cell nucleus. The mature micro-gametes leave their host cell and fertilize the 5µmx5µm large macro-gametes. These also are nearly spherical and can be differentiated from other developmental stages by their granulated cytoplasm and their eccentrically positioned wall-forming bodies. By syngamy, the only diploid *Cryptosporidium* development stage, the zygote, develops. It subsequently carries out a meiosis-similar process, called sporogony. Still within the parasitophore vacuole, thin- or thick-walled oocysts with 4 haploid sporozoites each (sporulated oocysts) develop (O'Hara and Chen, 2011). Thin-walled oocysts (about 20%) already excystate within the intestinal tract of the host and are believed to cause an endogenous auto-infection, whereas the thick-walled extremely resistant oocysts (about 80 %) are released with the faeces into the environment. They represent the only exogenous stage of the *Cryptosporidium* parasite.

The recent review of Lendner and Daugschies (2014) summarizes in detail the molecular components and mechanisms involved in the development cycle of *Cryptosporidium.*

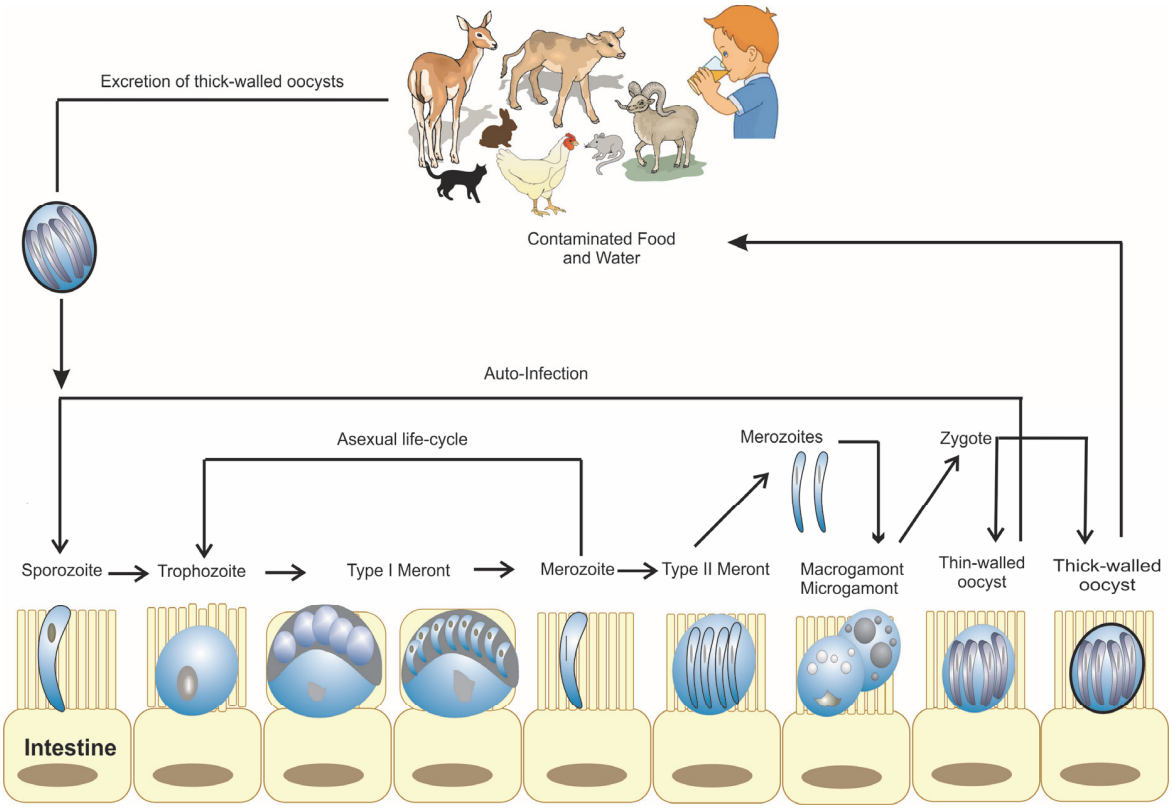

Figure 1: Life cycle of *Cryptosporidium* in different animal species and humans

2.1.4 Host spectrum

Currently, 26 morphologically, biologically and molecular-biologically confirmed different *Cryptosporidium* species are listed (Fayer and Santin, 2009; Elwin et al., 2012; Lebbad et al., 2013; Adamu et al., 2014), having mammals (primates, bovidae, equidae, carnivora, hares, rabbits, tapiridae and rhinocerotidae), amphibians, reptiles and birds as hosts.

(Fayer, 2008) listed 157 mammalian species as hosts. Tzipori and Ward (2002) stated that *Cryptosporidium*, further to all four classes of vertebrates, most likely infect all mammalian species. Major species found in mammals are: *C. andersoni, C. bovis, C. canis, C. fayeri, C. felis, C. hominis, C. macropodum, C. muris, C. parvum, C. ryanae, C. suis* and *C. wrairi.* The three originally from birds isolated species are *C. baileyi, C. galli* and *C. meleagridis*, whereas *C. serpentis*, and *C. varanii* originate from reptiles and *C. fragile* from amphibians.

Primarily, *C. parvum* is considered the zoonotic species of human cryptosporidiosis; infection in most cases is due to water contaminated from animal faeces and/or due to contact with animals. Further human cases are caused by *C. hominis* via the anthroponotic

pathway (Lendner et al., 2011). A true host specificity of the species in most cases, however, does not exist and after *C. baileyi, C. canis, C. felis, C. meleagridis, C. bovis, C. suis, C. andersoni* and *C. muris* also were detected in cases of human cryptosporidiosis, these species, further to *C. parvum*, also have to be considered potentially zoonotic (see: Helmy et al. (2013)). The zoonotic potential of these species has to be judged as lower, although not in immunosuppressed persons (Lendner et al., 2011).

Furthermore, more gene sequence data exist for more than 40 isolates of vertebrates; as these isolates so far could not be allocated taxonomically, they are listed only as host-referred-to genotypes in the literature (Fayer, 2010).

Table 1: Differences among *Cryptosporidium* species: their major hosts, oocyst sizes and locations in the gut: (modified after Sunnotel et al., 2006; Caccio and Widmer, 2014)

***Cryptosporidium* spp.**	**Hosts**	**Oocyst size (µm)**	**Location**
C. parvum	Ruminants, humans, deer	4.5 x 5.5	Small intestine
C. bovis	Ruminants	4.2-4.8 x 4.8-5.4	Small intestine
C. andersoni	Ruminants, camel	5.5 x 7.4	Abomasum
C. ryanae	Ruminants	3.2 x 3.7	Small intestine
C. hominis	Humans	4.5 x 5.5	Small intestine
C. meleagridis	Birds, humans	4.5-5.0 x 4.6-5.2	Intestine
C. baileyi	Birds	6.4 x 6.2	Cloaca, bursa, respiratory tract
C. galli	Birds	8.0-8.5 x 6.2-6.4	Proventriculus
C. suis	Pigs, humans	5.1 x 4.4	Small intestine
C. wrairi	Guinea pigs	4.0-5.0 x 4.8-5.6	Small intestine
C. muris	Rodents, humans	5.6 x 7.4	Stomach
C. canis	Canids, humans	5.0 x 4.7	Small intestine
C. felis	Felids, humans	4.5 x 5.0	Small intestine
C. molnari	Fish	4.7 x 4.5	Stomach
C. scophithalmi	Fish	3.0-4.7 x 3.7- 5.0	Intestine
C. nasorum	Fish	4.3 x 3.2	Intestine
C. saurophilum	Lizards, snakes	4.2-5.2 x 4.4-5.6	Intestinal and cloacal mucosa
C. serpentis	Snakes, lizards	4.8-5.6 x 5.6-6.6	Stomach
C. fayeri	Red Kangaroo, Marsupials	4.9 x 4.3	Intestine
C. macropodum	Marsupials	4.9 x 5.4	Small intestine
C. xiaoi	Sheep	3.9x3.4	Small intestine
C. ubiquitum	Sheep/wildlife	5.2x4.9	Small intestine
C. cuniculus	Rabbits	5.9 x 5.4	Small intestine
C. tyzzeri	Mice	4.6x 4.2	Small intestine

2.1.5 Genotypes/Subtypes

Recently, more than 60 *Cryptosporidium* genotypes which differ in their molecular sequences were listed (Nichols et al., 2010). In *C. parvum*, 10 subtype families (IIa, IIb, IIc, IId, IIe, IIf, IIh, Iii, IIj, IIk) have been identified (Table 2) with at least 78 subtypes. In *C. hominis*, 6 subtype families have been identified (Ia, Ib, Id, Ie, If and Ig) (Table 2) with at least 78 subtypes (Valenzuela et al., 2014).

Analysis of differences between species was developed for *C. parvum* and *C. hominis,* being the two most frequent species that cause human infection. A number of highly preserved genes have been targeted for this purpose, including small subunit rRNA (18S rRNA), 70 kilo Dalton (kDa), heat shock protein (HSP70), *Cryptosporidium* oocyst wall protein (COWP) and the actin gene. The 18S rRNA gene is useful because in addition to regions that vary between species, it contains several regions that are conserved within the *Cryptosporidium* genus. This makes it easy to develop primers that target most species.

DNA extracted from oocysts can be amplified by one of several methods, including standard or nested polymerase chain reaction (PCR) protocols. In standard PCR, one pair of primers is used to amplify a gene in the forward (5'-) and reverse (3'-) directions, whereas in nested PCR two sets of primers are used, of which the first (external) primer pair targets the gene of interest. A second (internal) primer pair is then used to amplify a shorter (internal) segment of the amplicons produced in the primary PCR.

Mixed infections are hard to identify by PCR with 18S rRNA, kDa, HSP70, COWP and the actin gene. The GP60 gene is useful because the dominating species or the species with highest affinity for the primer will be amplified to a much larger extent than the other(s), resulting in identification of only the dominant species. GP60 subtype analysis will avoid this problem because the primer used is quite species specific (Silverlås, 2010).

The GP60 gene has a highly polymorphic region of microsatellites in the 5' end, consisting of trinucleotide repeats (TCA, TCG, TCT), all coding for the amino acid serine. *Cryptosporidium* subtypes are named according to the number of each present repeat; some subtypes have other short repetitive sequences (R) immediately after the trinucleotide repeats. *G. hominis* subtype families have prefixes Ia, b, and d-g, whereas *C. parvum* subtype families have prefixes IIa-IIk.

Table 2: **Major GP60 subtype families and subtypes for *C. parvum* and *C. hominis* and their representative sequences** (Jex and Gasser, 2010; Xiao, 2010)

Species	Subtype family	Subtype	Dominant trinucleotid repeat	GenBank Accession Number
C. parvum	IIa	A15G2R1	TCA, TCG	AY262034
		A15G2R2		DQ192501
		A16G2R1		EU877254
		A17G1R1		EF576978
		A17G2R1		EF576964
		A17G2R2		JX575590
		A17G3R1		JX575588
		A18G2R1		HM370437
		A18G3R1		EU379548
		A19G2R1		EF576962
		A19G3R1		JQ362496
		A20G2R1		KC291661
		A20G3R1		EU164809
		A21G3R1		EF576958
		A22G3R1		JQ518593
	IIb	A14	TCA	AF402285
	IIc	A5G3a	TCA, TCG	AF164491
		A5G3b		AF164501
		A5G3c		EU095267
		A5G3d		AF440636
	IId	A17G2R1	TCA, TCG	
		A18G1		AY738194
		A18G2R1		
		A19G2R1		
		A22G2R1		EU877259
	IIe	A12G1	TCA, TCG	AY382675
	IIf	A6	TCA	AY738188
	IIg	A9	TCA	AY873780
	IIh	A7G4	TCA, TCG	AY873781
	IIi	A10	TCA	AY873782
	IIj	A18	TCA	AM937006
	IIk	A14	TCA	AB237137
C. hominis	Ia	A23R4	TCA	AF164502
	Ib	A10G2	TCA,TCG, TCT	AY262031
		A9G3		DQ665688
		A10G2R2		EU877240
	Id	A16	TCA, TCG	DQ665692
		A15G1R1		
	Ie	A11G3T3	TCA,TCG, TCT	AY738184
	If	A19G1	TCA, TCG	AF440638
	Ig	A24	TCA	EF208067

2.1.6 Infection mechanism

After the host has ingested the infective stage (oocyst) with food or water, the parasite expresses many molecules on the sporozoite surface that mediate attachment and probable invasion. Infection of host cells is initially a parasite-driven process, but the signaling events and their downstream actions within *Cryptosporidium* are poorly understood.
Calcium likely is one of the important second messengers responsible for signal transduction, but only a few kinases and transporters have been addressed so far (Lendner and Daugschies, 2014). Calcium-dependent protein kinases (CDPKs) are probably involved in the regulation of invasion and egress (Etzold et al., 2014). Lendner and Daugschies (2014) mentioned that the application of experimental results from other coccidian parasites is not possible, because *Cryptosporidium* is special, in that it is embraced by the host cell instead of invading it, that it stays in an epicellular location and that it induces tremendous actin rearrangement in the cell.

The authors point to the analyses of genomic data of *C. parvum* and *C. hominis* showing a highly streamlined metabolism that lacks many pathways found in other apicomplexa and which depends on the import of essential nutrients from the host. Moreover, *Cryptosporidium* likely releases unique molecules from the micronemes, addressing different receptors/pathways in the target cell. Remaining questions include what mediators are released by the parasite, the receptions addressed, the ways nutrients are transported and what the molecular basis of egress is.

2.1.7 Epidemiology

Cryptosporidiosis is a worldwide distributed intestinal infection caused by parasites of the genus *Cryptosporidium*. The endogenous sporulated oocyst is the infectious stage which by an infected host in this form is already excreted in large amounts in the faeces (in experimental infections of calves up to $4x10^7$ oocysts per gram faeces were determined (Fayer et al., 1998a)), or in a respective case of a respiratory cryptosporidiosis are excreted with the bronchial exsudate and immediately contaminate the environment. Several factors play a role in the epidemiology, rendering the control of *Cryptosporidium* difficult (Dillingham et al., 2002). Oocysts not only are very resistant against environmental factors; only few of the conventionally used concentrations of chemical disinfectants against bacteria or viruses do show some efficacy against these permanent stages due to the hard, nearly impenetrable oocyst wall. Due to this reason, in Germany one of the anti-

coccidial listed preparations of the disinfection lists published by the German Veterinary Society (Deutsche Veterinärmedizinische Gesellschaft (DVG)) is recommended for disinfection, even though oocysts of *Eimeria tenella* and not of *Cryptosporidium* are used as test organisms to test the anti-parasitic efficiency in regards to Coccidia.

Because of the small size of the oocysts, it is difficult to filter them from contaminated drinking water. Zoonotic transmission through direct contact or drinking of contaminated water can easily take place. The infective dose is very low, about nine oocysts of one *Cryptosporidium* isolate can cause infection of humans (Moore et al., 2003) and about 50 oocysts in calves (Okhuysen et al., 1999). It has been suggested that during the Milwaukee outbreak the infectious dose of *Cryptosporidium* may have been 1 to 10 oocysts for some persons (Dillingham et al., 2002). In contrast, one infected host can shed as many as 10^{10} oocysts, contributing to a huge infection pressure. The oocysts are sporulated and infectious at shedding, which means that a new host can immediately be infected.

2.1.8 Risk factors for infections with *Cryptosporidium*

Calves usually become infected by the oral uptake of oocysts from the environment. Possible major sources of infection, next to infected and shedding neighbor animals, are contaminated stables, faeces and dirty teats and udders of suckling cows. Of particular importance are clinically inapparent diseased animals which, despite lacking clinical symptoms, can serve as shedders of oocysts. Sub-clinical infections not only were detected in calves, but also in adult cattle (Villacorta et al., 1991; Lorenzo Lorenzo et al., 1993), so that this later age group cannot be excluded as a potential agent reservoir. Oocysts further can be acquired by animal handling personnel via dirty shoes, clothes, wheelbarrows, etc. as well as by infected and externally contaminated rodents, dogs, cats, wild animals, insects (flies, cockroaches, dung beetles) and by free-living amoeba. Together with enterotoxic *E. coli*, Rota- and Corona-viruses, *Cryptosporidium* is one of the major causes of neonatal diarrhea of calves. Reported prevalences of bovine cryptosporidiosis range between zero and one hundred percent, with prevalence tending to decrease with increasing age (Santin et al., 2008). Studies from the US furthermore account for an age-dependent species variation in *Cryptosporidium* infected calves. In up to 8 week old calves, *C. parvum* is the dominant species, whereas in 2-to-11 month old calves *C. bovis*, a host-adapted species, originally not associated with an actual clinical course, is predominant (Fayer et al., 2007; Santin and Trout, 2008).

Factors leading to *Cryptosporidium* infection of humans are multiple (Lendner et al., 2011). Outbreaks often are due to contaminated water, both drinking water, but also unclean recreational/ swimming pool water. Further means of distribution are contaminated foods, like raw fruits and vegetables fertilized with contaminated effluent, contact between people (hospitals, day care centers, schools), aerogen transmission and contact with animals (particularly calves). Transmission by anal sexual exposure is possible (Dillingham et al., 2002).

2.2 Cryptosporidiosis

2.2.1 Clinic of human cryptosporidiosis

The clinical course of cryptosporidiosis strongly depends on the age and the immune status of the respective patient (Tzipori, 1988; Chen et al., 2002). In immunocompetent patients, the incubation period of about 5 to 21 days is followed by an acute self-limiting diarrhea, which lasts from 3 to 12 days. The main symptom of the medium to strong profuse, watery to catarrhal diarrhea often is accompanied by abdominal cramps, nausea, vomitus, flatulence, bloating, anorexia and fatigue. Asymptomatic courses though also are possible (Current and Garcia, 1991; Farthing, 2000). In contrast to the self-limiting causes of disease in immunocompetent patients, cryptosporidiosis in immunodeficient persons can develop into a chronic and life-threatening disease (Farthing, 2000). Particularly affected are people whose T-cell function as a consequence of a genetic immune defect (e.g. hyper-IgM-syndrome) is compromised or whose numbers of CD4-lymphocytes by acquired causes (e.g. HIV infection, immunosuppressive therapy after organ transplantation) are markedly reduced (Hunter and Nichols, 2002); the number of CD4 cells seems to be decisive for the severity of the disease.

In this group of people, also extra-intestinal forms of cryptosporidiosis can occur. That way, *Cryptosporidia* have been detected in the gallbladder system and in the respiratory tract of HIV/AIDS patients. The colonization of the respiratory tract does not have to be associated with clinical pain (Hunter and Nichols, 2002). Also, in patients suffering from primary severe combined immune deficiencies (SCID), which result in an insufficiency of the cell-mediated immunity, extra-intestinal forms do occur (in respiratory bronchioles and the ductus pancreaticus a.o.).

The question whether e. g. *C. parvum* could be involved in the genesis of tumor cases constitutes the work of some research groups. In a rodent model, a carcinogenic potential of 2 *C. parvum* lines of animal origin (IOWA and TUM1) could be demonstrated.

Dexamethason-treated SCID mice in the cause of an experimentally induced infection developed neoplasias with invasive character in the gastrointestinal tract (Certad et al., 2010a; Certad et al., 2010b). Recently, this cause could be verified also for a line of human origin (IIaA15G2R1 genotype), whereby additionally an induction of a bile duct carcinoma could be observed (Certad et al., 2012).

In children and HIV-positive persons, differences in clinical manifestations have been observed between *C. hominis* and *C. parvum*, with *C. hominis* being more virulent than *C. parvum* (Hunter et al., 2004; Cama et al., 2007). Infections with *C. parvum* were associated with chronic diarrhea and vomiting in HIV-positive persons more frequently than infections with *C. hominis* (Cama et al., 2007).

Infection at a young age can lead to impaired development and growth, and possible long-term cognitive deficits especially among children in the developing world (Dillingham et al., 2002).

2.2.2 Clinic of animal cryptosporidiosis

In animals, cryptosporidiosis is mainly observed in young calves and the severity of the disease depends on several factors such as host immunity, infective dose and current infection with other pathogens such as rotaviruses. Symptoms vary from asymptomatic to pasty or watery profuse diarrhea, dehydration and mortality.

C. parvum, together with enterotoxic *Escherichia coli* and Rota- and Corona-viruses, is an essential component of the calf diarrhea complex (diarrhea within the first three weeks) and by this is one of the major causes of losses in calf rearing.

The clinical picture of the neonatal diarrhea within a mono- or also mixed-infection with *C. parvum* is characterized by profuse, yellowish diarrhea and its consequences, like exsiccosis, metabolic acidosis and loss of electrolytes (Tzipori et al., 1983; Kaske et al., 2008). These become manifest in form of caved in bulbi, decreased skin turgor, coolish acra and general infirmity.

The economic losses are short-term due to treatment costs of the diarrhea and longer term due to significant morbidity, impairment of growth, reduced weight gain and increased mortality in diseased animals (De Graaf et al., 1999; McDonald, 2000).

Clinically manifest bovine cryptosporidiosis is limited to neonates (Harp et al., 1990). The extent and degree of the disease are likely dependent on an acquired immunity by a preceding exposition with very low infective doses and on the age of the animal and its accompanying maturation of the immune system *per se* (Current and Garcia, 1991).

2.2.3 Pathological findings/pathophysiology

The pathogenic mechanisms of *Cryptosporidium* causing diarrhea, malabsorption and wasting are poorly understood. The initial host–parasite interactions due to attachment and invasion of *Cryptosporidium* are critical primary events in the pathogenesis (Tzipori and Ward, 2002).

C. parvum in calves causes acute to chronic catarrhal enteritis primarily in the distal ileum, but parasite stages also were detected in the duodenum and in portions of the caecum and the colon. The mucosa of affected areas is hyperemic and edematous (Fayer et al., 1990b). The mesenteric lymph nodes are partially enlarged and also edematous. Histological findings include mild to moderate villus atrophy as well as occasional villus fusion. The crypts of affected areas partly are dilated and contain debris and neutrophil granulocytes. Neutrophil granulocytes and a massive mononuclear cell infiltration (among others macrophages) also were proven in the lamina propria mucosa in histopathological investigations conducted by Tzipori et al. (1983).

A *Cryptosporidium* infection in the calf furtheron leads to versatile alterations of the surface epithelium: cell degeneration, metaplasia of physiological high prismatic to isoprismatic villus epithelial cells, hyperplastic crypt epithelium, displacement of microvilli in the area of the attachment zone of the intracellular parasite stages and unusually long microvilli directly in the neighborhood of the parasite stage (Heine et al., 1984).

The aforementioned pathological alterations result in a diminution of the total absorption-active intestine surface and consequently are accompanied by malabsorption. The hyperplastic crypt epithelium is an expression of a compensatory attempt to replace the damaged villus epithelial cells. The replacement enterocytes from the proliferative zone though still are not differentiated and thus overwhelmingly only have secretory capacity. In consequence of their chloride secretion, water from the blood is shifted into the intestine lumen.

The damages of the intestinal epithelium additionally lead to a reduced activity of the brush border membrane enzymes (glocoamylase, alpha-dextrinase, saccharase, lactose), whereby the digestive capacity for carbohydrates in the small intestine is exceeded; this circumstance can only partially be compensated by increased microbial decomposition of carbohydrates to short-chain fatty acids in the small intestine. Subsequently, osmotically

effective particles remain in the gut lumen; water resorption is impaired and an osmotic diarrhea develops.

Additionally, as an immunologic response to the membrane damages, prostaglandins are synthesized by enterocytes of intra- and sub-epithelial lymphocytes, plasma cells and macrophages, which themselves, next to increased blood vessel permeability and pains, induce an escalated chloride secretion into the gut lumen (Tzipori et al., 1983).

The formation of a cholera-like toxin by *Cryptosporidia* as an triggering agent of a secretory diarrhea also has been discussed (Angus, 1990). To date, no such toxin, also named Sekretagogum, has been verified (Warren and Guerrant, 2008).

In humans, the pathophysiological mechanisms of diarrhea as a main symptom in a *Cryptosporidium* infection also are not completely clarified. Case studies either reported a secretory or an osmotic diarrhea in *Cryptosporidium* infected HIV/AIDS patients (Warren and Guerrant, 2008).

2.2.4 Public health significance

Infections of the human gastrointestinal tract with enteric pathogens are among the leading causes of disease, suffering, and death worldwide. Particularly, more than 58 million cases of diarrhea detected per year in children are associated with intestinal protozoan infections with high morbidity and mortality rates. Particularly *Cryptosporidium* and *Giardia* are major causes of diarrheal diseases in humans worldwide and are included in the "World Health Organization's Neglected Disease Initiative" (Savioli et al., 2006). Waterborne contamination is a growing concern causing widespread disease outbreaks.

Cryptosporidium now additionally is considered an important food-borne pathogen causing a disease of socioeconomic significance worldwide (Putignani and Menichella, 2010). Factors that have contributed to the emergence of cryptosporidiosis in animals include increased environmental contamination and trends in livestock production. In developing countries the impact of protozoan pathogens represents a major cause of gastrointestinal illness and is becoming of growing impact. *Cryptosporidium* accounts for up to 20% of all cases of childhood diarrhea in developing countries and is a potentially fatal complication of AIDS (Mosier and Oberst, 2000). However, a large proportion of illnesses in these countries, especially in children, are still ascribed to an unknown etiology. The diseases are often indicators of poverty and disadvantage, the most affected are the poorest populations often living in remote rural areas, urban slums, conflict and natural disaster zones, where aggravate conditions are conducive to the spread of these

diseases (Putignani and Menichella, 2010). Repeated or prolonged episodes of diarrhea in early childhood are often associated with poor cognitive function, failure to thrive and increased risk of stunting.

In pediatric populations, prevalence still is grossly underestimated, due to a poor clinical valuation of pathognomic symptoms and the absence of advanced laboratory tools in diagnostic routine panels.

2.2.5 Prevention and control

As long as all *Cryptosporidium* infections are caused by ingestion or inhalation of oocysts, and due to no effective treatment, measures to prevent or limit the spread of infections must content themselves with the targeted elimination or reduction of contaminations with infectious oocysts in the environment (Fayer and Xiao, 2007).

In animal populations, continuous movement of animals and cleaning of areas are recommended, but possibly not economical with large numbers of animals. For humans, better disinfection is said to minimize person-to-person transmission in domestic and institutional settings. A high priority has to be given to measures that effectively address the contamination of recreational and drinking water.

Various physical stresses such as heat, cold, irradiation, pressure, and desiccation affect *Cryptosporidium* oocysts. Their survival time is shortened if the temperature decreased below 5°C or increased above 15°C (Fayer et al., 1998b). The aging of *C. parvum* oocysts at different temperatures is linked to their carbohydrate energy reserves stored in the sporozoites, and the residual bodies such as amylopectin granules are consumed more rapidly at higher temperatures (Jenkins et al., 2003). Amylopectin assists in the excystation process and host-cell invasion (Fayer and Xiao, 2007). Increasing temperatures to 64.2°C or more for 5 min and 72.4°C for 1 min returns the oocysts non-infectious (Fayer, 1994).

C. parvum oocysts survive freezing at –20°C for extended periods, but not at –70°C or lower, even in the presence of cryoprotectants (Fayer, 1994). Ultraviolet (UV) irradiation has an effect on rendering oocysts non-infectious (Sivaganesan and Sivaganesan, 2005).

Among the commercial disinfectants that appear most effective are those that contain hydrogen peroxide, chlorine dioxide, or ammonia. Although bromine-, chlorine-, and iodine-related compounds can greatly reduce the ability of oocysts to excyst or infect,

relatively high concentrations or long exposure periods are required, limiting most practical applications.

Ozone seems to be one of the most effective chemical disinfectants and has an application against oocysts in water (Fayer and Xiao, 2007).

Under experimental conditions rotifers, which occupy niches in seawater, in rivers, lakes and ponds, and predacious protozoa have been found to ingest oocysts of *C. parvum* (Stott et al., 2003). Shellfish, including oysters, clams, mussels, and cockles are filter feeders that remove small particles from the surrounding aquatic environment. Oocysts have been detected in gills and in the digestive diverticula of shellfish in numerous freshwaters and tidal and coastal water locations in North America and Europe (Fayer et al., 2003; Fayer, 2004).

2.2.6 Chemotherapeutics

In the last years, up to 100 active components have been tested for their suitability as an anti-cryptosporidial chemotherapeutic (Stockdale et al., 2008). Although several pharmacological compounds in *in vitro* studies showed an anti-cryptosporidial activity, considerably fewer demonstrated a significant potential in animal experiments and many compounds with initially positive results ultimately were ineffective or only partially effective (Mead, 2002; Stockdale et al., 2008).

A causal medical treatment of a patent bovine cryptosporidiosis to date is not available, despite numerous studies and testing of diverse active components (Shahiduzzaman and Daugschies, 2012). In Europe, only a single active ingredient, Halofuginone, a bromo-chlorinated chinazolin- derivate, is approved for pro- and metaphylactic treatment. It has to be applied therapeutically for 7 days, starting within the first 24 hours after the onset of the diarrhea symptomatology, prophylactically within the first 24 to 48 hours of life. An exact adherence to the dosage of 100 µg/kg body weight is necessary as the therapeutic index of the active ingredient is small; symptoms of poisoning in the form of diarrhea, blood in faeces, reduced milk intake, dehydration, apathy and exhaustion already can appear at a double therapeutic dosage (Silverlas et al., 2009).

Nitazoxanide, a nitrothiazolylsalicylamide was approved for the treatment of human cryptosporidiosis, for example in 2005 by the US “Food and Drug Administration” (FDA). The efficacy of Nitazoxanide without an efficient immune system (number of CD4 cells) seems to be limited, several authors therefore only attest a partial efficiency (Cabada and White, 2010).

2.2.7 Water treatment

About 56% of the 71 *Cryptosporidium*-linked outbreaks in the last decade appear to be correlated to waterborne diseases and worldwide environmental and veterinary surveillance data revealed the presence of *Cryptosporidium* spp. in entire wastewater, surface water and water-treatment systems (Putignani and Menichella, 2010). Therefore, control of the parasite is a major challenge to water treatment professionals. *Cryptosporidium* testing has a role, but the prevention of cryptosporidiosis as waterborne disease is the result of proper water treatment, not pathogen testing. Infectious oocysts pass through different filtration processes and are unaffected by chlorine and chlorine-based disinfectants; outbreaks occur even in water from plants meeting all water quality and operational standards. In developing countries, different filtration methods (conventional, direct, slow-sand, diatomaceous earth, bag or cartridge, membrane) will have to be introduced or made effective in nonfunctional water purification stations and ineffective water treatment systems.

Conventional filtration with a process of coagulation, flocculation, sedimentation and filtration may be capable of 99% removal of *Cryptosporidium* (Fayer and Xiao, 2007). Direct filtration is similar to conventional filtration in that a coagulant is used to form larger particles, but the settling or sedimentation step is not included. Sand filtration uses a biological process to remove organic contaminants from the source water.

A biological ecosystem forms on top of a sand filter and dissolves organic material as it passes through in the water. In diatomaceous earth filtration, a depth filter is developed after each backwash by applying granular particles of a specified size-distribution to a backing screen while operating the filter in a recirculating mode. In a bag or cartridge filtration, bag filters, being sheets of filter material, are folded to form a pocket or bag in the direction of water flow. Cartridge filters consist of surface or depth filter media wrapped or constructed around a hollow core, where the feed water contacts the outer perimeter and, with pressure, moves across the filter to the core. In membrane filtration processes, water is forced by pressure across a surface, excluding or rejecting particles greater than the effective pore size of the membrane material. Membrane filtration processes are classified as reverse osmosis, nanofiltration, ultrafiltration, and microfiltration, with filter porosity correspondingly increasing from the molecular to the micrometer level.

Oocysts are very sensitive to inactivation by UV irradiation (Rochelle et al., 2004; Johnson et al., 2005) and it has been suggested that sunlight may be the most significant inactivating agent in environmental waters (Caccio and Widmer, 2014).

2.3 Diagnostics

2.3.1 Diagnosis of *Cryptosporidium*

2.3.1.1 Parasitological diagnosis

Several methods exist to detect *Cryptosporidium* in fecal samples. The most common method is microscopy for the detection of oocysts. Fecal samples can be examined directly on slides or after concentration either by flotation or sedimentation to remove fecal debris or to concentrate the number of oocysts; the detection of oocysts in animals with low numbers of oocysts is facilitated (Fayer and Xiao, 2007).

Visualization of *Cryptosporidium* oocysts by microscopy most commonly is done by direct smear and without any staining and by the modified Ziehl-Neelsen stain under light microscopy, whereby the oocysts stain purple with blue background. Immunofluorescence staining techniques using monoclonal antibodies against the oocyst wall antigen under epi fluorescence microscopy are also useful. Immunofluorescent antibody-based (IFA) procedures have a high sensitivity, but still the easier and cheaper traditional staining methods such as the Ziehl-Neelsen stain are widely used, despite their lower sensitivity (Caccio and Widmer, 2014).

Most parasitological detection methods for *Cryptosporidium* do not distinguish between viable and nonviable oocysts.

2.3.1.2 Serological diagnosis

Serological methods are particularly useful tools for screening of large numbers of samples, like in epidemiological surveys. Most serological tests used to identify exposure/infection are enzyme linked immunosorbent assays (ELISA) or enzyme-linked immunoelectrotransfer blots (EITB; Western blot) employing various aqueous extracts of *C. parvum* oocysts.

Enzyme immunoassay (EIA) methods are fast, inexpensive, easy to be performed, and show sensitivity comparable to that of the immunofluorescence methods (Fayer and Xiao, 2007). Rapid immunochromatographic (strip) tests can be also used. These tests rely on the detection of cell wall proteins of the oocysts using monoclonal antibodies (Papini and Cardini, 2006).

2.3.1.3 Molecular diagnosis

Several nucleic acid detection techniques are described for the detection of *Cryptosporidium*, some of which may be able to distinguish viable from nonviable oocysts. Tests include single-round and nested PCR, random amplified polymorphic DNA PCR (RAPD-PCR), arbitrary primed PCR (AP-PCR), reverse transcription PCR (RT-PCR), real-time PCR, followed by restriction fragment length polymorphism (RFLP) analysis, single strand conformation polymorphism (SSCP) analysis, melting curve analysis, microarray and DNA sequencing (Egyed et al., 2002). Detection of cryptosporidiosis using PCR-based methods is more sensitive than by conventional microscopical and serological methods for detecting oocysts in faeces. Molecular methods can also identify the species/genotypes and subtypes of *Cryptosporidium*, important for determining the epidemiology of *Cryptosporidium* and predicting transmission routes (Caccio et al., 2005). Since the description of the first PCR-based tool for the differentiation of *C. parvum* and *C. hominis* (Morgan et al., 1995), molecular techniques in the diagnosis of cryptosporidiosis became popular, especially due to their genotyping capabilities. The target genes of *Cryptosporidium* that can be detected using molecular diagnosis include, but are not limited to, 18S rRNA, KDa, HSP70, COWP and the actin gene. Specific PCR assays targeting the 18S rRNA gene are very useful for the detection (targeting a conserved region in the gene) or differentiation between *Cryptosporidium* spp. (targeting variable nucleotide stretches) (Fayer and Xiao, 2007).

RFLP is used for species differentiation; restriction enzymes are used to digest amplicons in fragments of varying size, depending on the species, that cause the products to migrate in different distances on the gel (Ghaffari et al., 2014). Another method to determine different species is by direct DNA sequencing using DNA, purified and amplified by internal primers and labeled with colored nucleotide bases which omit light at different wavelengths; this property is used to analyze the gene sequence (Morrison et al., 2008). The produced forward and reverse sequences can be assembled to contigs and compared to the deposited sequences in the Gene Bank using BLAST (Basic Local Alignment Search Tool; http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Mixed infections are not easily identified using PCR, where the dominating species or the species with the highest affinity for the primers will be amplified to a larger extend than the others; this may result in the identification of only the dominant species. If more than one species amplifies successfully, this is indicated at gene sequencing as double peaks in many positions and thereby inability to assemble contigs. For successful analysis of mixed

infections, either a combination of several species/genotype-specific primers (Xiao and Fayer, 2008) or cloning of single amplicons produced in the area have to be used. Another possibility is to perform GP60 subtype analysis because the primer used is quite species-specific.

GP60 is expressed on the apical surface of invading stages (sporozoites and merozoites) and is targeting for neutralizing antibodies (Cevallos et al., 2000). GP60 subtyping can particularly help to determine the virulence of different *C. parvum* and *C. hominis* subtypes.

2.4 Egypt

2.4.1 *Cryptosporidiosis* situation in different provinces of Egypt

2.4.1.1 Situation in animals

In Ismailia province, the prevalence of *Cryptosporidium* was estimated to be 15.4% in calves, 9.9% to 13.3% in buffalo-calves and 25% in camel calves (Abou-Eisha, 1994; Abou-Eisha et al., 2000). Other studies estimated 20.9% prevalence in sheep, 22.5% in buffaloes, 23.7% in cows, 25.9% in goats, 2.6% of dogs and 6.3% in wild rats (Abou-Eisha, 1994; Abou-Eisha et al., 2000; Shoukry et al., 2009). Compared to Ismailia province, relatively higher prevalences in calves have been reported for other provinces in Egypt; 54.4% in the Beheira province (Hassanein et al., 2012), 30.2% in the Kafr El Sheikh province (Amer et al., 2010) and 14.2% in Dakahlia and Kafr El Sheikh provinces (El-Khodery and Osman, 2008). Moreover, the prevalence of *Cryptosporidium* in lambs ranged from 17.1% to 68.3% in Qalubia province (Abd El Wahed, 1999). The prevalence of *Cryptosporidium* was 31.9% in quails, 50% in dogs and 22.7% and 20.3% *C. muris* were identified in wild rats in Qalubia province of Egypt (Abd El Wahed, 1999; Shaapan et al., 2011).

An evaluation of the *Cryptosporidium* situation in animals in Egypt from these studies is difficult, if not impossible. Sampling schemes used, if at all, numbers of samples collected and, most importantly, what diagnostic methods were used, vary too much to permit generalization or abstraction from the studies.

2.4.1.2 Situation in humans

The prevalence of *Cryptosporidium* in children in Ismailia province was estimated as 33.3% (Shoukry et al., 2009), 3.6% in Damietta province (Samn et al., 2012), 4.6% in ten

public hospitals in Cairo province (Abd El Kader et al., 2012), 31.1% in out- and in-patients from different areas in Cairo province (Mousa et al., 2010), 9.5% in Mansoura (El-Shazly et al., 2007b), 15% in children from Fayoum, 17% among diarrheic children of the Nile River, 48.8% in Alexandria province (Allam and Shehab, 2002), 16.4% and 21.3% respectively, in kids in Ismailia province (Abou-Eisha, 1994; Abou-Eisha et al., 2000), 13.51% in school children in rural areas in Alexandria province (Soliman, 1992) and 23.8% in cancer patients. In contrast, it was 37.7% and 91% in children and adult immunodeficient patients in general Egypt (Hassan et al., 2002), 19.5% in diarrheal patients of all age groups attending the outpatient clinics in Qaluibia province (Abdel-Maboud et al., 2000), 9% in children with diarrhea in Aswan province (Mikhail et al., 1989), 60.2% in Minia province (Abdel-Hafeez et al., 2012), 29% in children suffering from diarrhea (El-Naggar et al., 1999) in Dakahlia province, 33.3% in immuno-compromised children in Tanta province (Antonios et al., 2010) and 15% in El-Sharkiya province (Ali et al., 2000).

As with animal cryptosporidiosis, there is interest in the Egyptian medical research community on *Cryptosporidium*. Most studies though bear the same shortcomings in regards to hypothesis development as noted for the animal studies.

Figure 2: Map of Egypt with provinces

1-Demiat Province, 2-Kafr El Sheikh Province, 3-Dakahlia Province, 4-Gharbia Province, 5-Menufia Province, 6-Beheira Province, 7-Alexandria Province, 8-Sharkia Province, 9-Qalubia Province, 10-6 of October Province, 11-Cairo Province 12-Suez Province, 13-Helwan Province, 14-Fayoum Province, 15-Beni Sweif Province, 16-Menia Province, 17-Assiut Province, 18-Sohaj Province, 19-Qena Province and 20-Aswan Province

2.4.2 Animal populations and husbandry in the Ismailia province of Egypt

Egypt has limited natural pasture and animal production is highly dependent on cattle and buffaloes for milk-production, whereas males and non-reproductive females are fattened for meat production. In Egypt, livestock numbers in the period between 2000 and 2009 did increase, particularly the cattle population (from 3.53 to 5.00 million), buffaloes (3.38 to 4.00 million), goats (3.43 to 4.55 million) and sheep (4.47 to 5.50 million). Camels, in contrast, have declined from 141,000 to 110,000 heads (El-Nahrawy, 2011). In 2009, numbers declined, mostly due to Foot and Mouth Disease (FMD), Lumpy Skin Disease (LSD) and Three Days Fever (USDA, 2010). Recent animal population data are not available.

The respective numbers of cattle, buffaloes and humans in the Ismailia province study area are contained in Table 3.

Table 3: Distribution of animal and human populations in Ismailia province of Egypt (Ministry of Agriculture, personal contact directly from the office)

Area	Animal populations		Total	Human population
	Cattle	Buffaloes		
Ismailia	9170	6400	15570	465262
El Tal- Elkabeer	15600	7200	22800	169081
Kassasin	5100	3800	8900	
Abu swair	8500	3100	11600	133170
Qantara gharb	6350	3900	11250	117622
Fayed	4100	3450	7550	122600
Qantara shark	2400	1200	3600	46147
Total	51220	29050	80270	1053882

As more than 80% of Egypt is desert, the Nile River and systems of irrigation canals have to provide water for agricultural production. In the Ismailia province, there are dense populations of humans, cattle and buffaloes sharing the narrow banks of the Suez Canal and a system of lateral canals. The smallholder extensive animal system represents the majority of animal keeping systems in the province. It was estimated that around 11,000 farms in Ismailia province belong to the smallholders category (EFARP, 2004).

Smallholder farmers, not owning agricultural lands or controlling agricultural holdings, are the main source of animal production (El-Nahrawy, 2011). They are traditional farmers, literacy is low and many of the current farmers do not have a farming

background. About 89% of cattle and 75% of buffaloes are kept in holdings of less than 2.1 ha; about 93% of cattle and 86% of buffaloes constitute herds of less than ten animals. Women are traditionally in charge of the animals. They are responsible for the daily animal care, milk production, and decisions over its consumption and sale, while their husbands decide on the sale of animals and use of the income. The animals are kept in the home compounds, tied either in a barn constructed from locally available materials or in corrals near the farmer's house. During the day, they usually are tethered at the side of a field and are fed cut and carried available crop by-products. They often are watered with surface water or water from shallow wells. Cattle and buffalo are usually kept together in the same premises (El-Nahrawy, 2011).

3 Publications

3.1 Publication 1: Frequencies and spatial distributions of *Cryptosporidium* in livestock animals and children in the Ismailia province of Egypt
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Frequencies and spatial distributions of *Cryptosporidium* in livestock animals and children in the Ismailia province of Egypt

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SUMMARY

Faecal samples from 804 cattle and buffaloes and 165 diarrhoeal children of Ismailia province were investigated by an immunochromatographic screening test and PCR to determine prevalences and distributions of *Cryptosporidium* spp. Results were analysed statistically for clustering of animal and human cases. *Cryptosporidium* herd prevalence was 73·3% and individual animal prevalence 32·3%. *C. parvum* was the dominant species in animals (65·7%). Young calves watered with canal or underground water were at particular risk of infection. Detection rates were higher when calves showed diarrhoea, fever and dehydration. Human *Cryptosporidium* prevalence was 49·1%. *C. hominis* dominated in humans (60·5%), followed by *C. parvum* (38·3%). Living in villages, drinking underground water and having contact with animals were risk factors. Cluster analysis revealed differences in the distribution of infections between animals and humans and suggests different transmission dynamics.

Key words: Anthroponotic, *Cryptosporidium*, Egypt, epidemiology, zoonotic.

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Molecular epidemiology of *Cryptosporidium* in livestock animals and humans in the Ismailia province of Egypt

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ABSTRACT

The zoonotic potential of *Cryptosporidium* was studied in one of the most densely populated provinces of Egypt regarding livestock and people. In a representative survey, faecal samples from cattle, buffalo and stool samples from diarrhoeic children (<10 years) were investigated. Parameters assumed to be related to cryptosporidiosis were recorded for animals and children. Animal samples (804) were examined by the Copro-antigen *RIDA® QUICK* test, followed by PCRs targeting the 18S rDNA and *gp60* genes for antigen-positive and 10% randomly selected negative samples. All 165 human samples were tested by both methods. The overall estimated prevalence of *Cryptosporidium* in ruminants was 32.2%, without significant difference between animal species. PCR identified 65.7% *Cryptosporidium parvum*, 11.8% *Cryptosporidium ryanae*, 4.1% *Cryptosporidium bovis*, and combinations of *C. parvum* plus *C. ryanae* (11.2%), *C. parvum* plus *C. bovis* (5.3%) and of *C. parvum* plus *Cryptosporidium andersoni* (1.8%), also without significant differences in species occurrence between cattle and buffalos. The human *Cryptosporidium* spp. prevalence was 49.1%, of which 60.5% were *Cryptosporidium hominis*, 38.2% *C. parvum* and 1.2% *C. parvum* plus *C. bovis*. Analysis of *gp60* variants allocated *C. parvum* found in animals to the zoonotic subtype family IIa (18.9%, subtype IIaA15G1R1 only) and to IId (81.1%, mostly IIdA20G1). In humans 50% were classified as subtype family IIa (IIaA15G1R1 and IIaA15G2R1) and 50% were IIdA20G1. *C. andersoni* occurred only in cattle older than 1 year. In contrast, mono-infections with one of the three single *Cryptosporidium* species and the three combinations with *C. parvum* were more prevalent in cattle and buffaloes younger than 1 year, particularly in those younger than 3 months, and were predominantly subtype family IId. In human samples no *Cryptosporidium* were identified in children younger than 7 months. Neither place of residence nor the source of drinking-water had measurable effects on prevalence. Remarkably, however, all children with *C. parvum* subtype family IIa and 86% with subtype family IId had contact to animals. High prevalence and identical genotypes of *C. parvum* in animals and humans indicate zoonotic transmission due to contact with animals, involving IIdA20G1 as the most frequent subtype.

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4 Summary of the results of the dissertation

4.1 Publication 1:

Helmy YA, von Samson-Himmelstjerna G, Nöckler K and Zessin KH (2014). Frequencies and spatial distributions of *Cryptosporidium* of livestock animals and children in the Ismailia province of Egypt. Epidemiology and Infection, 1:1-11.

This study aimed to provide for the Ismailia province of Egypt valid estimates and interpretations on prevalences, distributions, risk factors and infection dynamics of cryptosporidiosis and involved *Cryptosporidium* species in livestock animals and in diarrheic children as a particular risk group of the human population, next to immunocompromised individuals. To the author's knowledge this is the first such geographic-related study on *Cryptosporidium* for Egypt.

As an analytic study, it, at least for the animal component, had to base on a probability planned sample. Best available information was the distribution of the total of about 6000 livestock herds in the 7 administrative districts of Ismailia province. A sample size of 190 herds to detect a prevalence of 50% with 95% confidence and 7% acceptable error was calculated and stratified random sampling was performed. Stratification was made for the respective numbers of herds in the seven districts Ismailia, Fayed, Kassasin, Abo Swair, El-Tal El Kabier, West Kantara and East Kantara. By this design, different district herd densities across and for the entire Ismailia province were represented in the sample. The ultimate herd sample consisted of 191 herds. At district herd level, individual sample herds were selected conveniently.

The within-herd samples of calves and diarrheic older animals (>2 years) would have consisted of 22 animals (44%) of a herd of size 50 animals to detect a herd prevalence of 10% randomly selected shedding animals with 95% confidence. As the study herds with an average of 7 animals (minimum 2, maximum 48) were much smaller, larger herd sample fractions of an average of 60% (minimum 33%, maximum 100%) were collected. These sample fractions were expected to provide valid estimates of the commonness of *Cryptosporidium* infection in the herd animals.

The sample of diarrheic hospitalized children by nature was non-random; whether and why a specific diarrheic child is presented and hospitalized in one of the 9 district hospitals of the province depends on many unaccountable circumstances. Consequently, the best possible way was to spread the calculated sample size of 156 children to detect a 50% prevalence with 95% confidence as wide as possible over the nine hospitals. At the

hospitals, the samples drawn were judgment or purposive samples. Eighty percent of the total of 165 children samples this way were collected, with 10 to 23 samples per hospital. The remaining 20% of samples were directly from households whose livestock animals were investigated.

Cryptosporidium positive animals were detected in 140 of 191 (73.3%) of herds and in 32.2% of individual animals in the positive herds. Whether herds consisted mainly of cattle or buffaloes did not affect prevalence. Shedders were detected in all age groups, young or old, but prevalence was significantly higher in the youngest calf age group of 1 day to 3 months.

Prevalence was essentially similar in all seven districts, with only Abo Swair and East Kantara districts showing higher values. Clinically, *Cryptosporidium* was more frequently detected in animals with watery and pasty feces. The source of drinking water was the only identified risk factor for animals; when animals were watered with canal or underground water, their risk of cryptosporidiosis was close to 2 times higher than when they were given tap water.

C. parvum single species infections (65.7%) dominated in the animals, fewer but still noteworthy numbers of cases of *C. ryanae* (11.8%) and *C. bovis* (4.1%) as sole species were further detected as were combinations of *C. parvum* with *C. ryanae*, *C. bovis* and *C. andersoni* at lower frequencies (11.2%, 5.3%, 1.8%, respectively). *C. ryanae* single infections and the *C. parvum* + *C. ryanae* combination relative to *C. parvum* increased in calves from 1 day age up to animals older than 2 years.

The *Cryptosporidium* prevalence of 49.1% in the diarrheic children was significantly higher than the 32.2% in individual animals. Children between 1.5 and 6 years were particularly affected, compared to younger and older ones. More children cases could be demonstrated in 2 of the seven districts. As with the animals, children had a higher risk of infection when they had drunk underground water, when they lived in villages and when they had contact with animals.

While *C. parvum* prevailed in animals, the majority of infections in children were due to *C. hominis* (60.5%). Moreover, 38.3% of infections were due to *C. parvum* and 99% of infections were strict single species infections due to either one of the two species. Only one species combination of *C. parvum* and *C. bovis* was detected in the 81 positive samples. Cluster analysis revealed differences in the spatial distribution of infections between animals and humans and suggests different transmission dynamics. Animal

cryptosporidiosis cases and *C. parvum* as major species are randomly distributed over the province. In humans, in contrast, clusters of cryptosporidiosis cases and those caused by *C. hominis* were identified around the Ismailia canal, the main sub-canal of the Suez Canal. Particular person-to-person hygiene risk factors in the handling of young children in these entirely rural districts are likely.

4.2 Publication 2:

Helmy YA, Krücken J, Nöckler K, von Samson-Himmelstjerna G and Zessin KH (2013). Molecular epidemiology of *Cryptosporidium* in livestock animals and humans in the Ismailia province of Egypt. Veterinary Parasitology 31;193(1-3):15-24.

In this study, the *Cryptosporidium* species of publication 1 were further investigated. Subtypes of *C. parvum* and *C. hominis* based on the sequence of the 60-kDa glycoprotein gene were determined and species and subtypes were analyzed for the uniqueness of their populations in categories of farm animals and humans. Molecular diagnostic results were co-analyzed with epidemiological information.

Analysis of *Cryptosporidium* by using Gp60 variants allocated *C. parvum* in animals to the zoonotic subtype family IIa (IIaA15G1R1) and IId (IIdA20G1), whereas those found in humans belonged to IIa (IIaA15G1R1 and IIaA15G2R1) and IId (IIdA20G1).

The high prevalence and the identical genotypes of *C. parvum* in animals and children are likely an indication for water contaminated by animals or zoonotic transmission due to contact of children with animals.

5 General discussion and conclusion

5.1 Prevalence of *Cryptosporidium*

Reported prevalence rates for *Cryptosporidium* species and genotypes reveal substantial variation between countries and amongst different geographical locations within a country due to the sampling design, type of populations studied, husbandry and management system, location, season of study, sanitary conditions inside and around farms and the used diagnostic methods (Ghenghesh et al., 2012). In Egypt, as an example, inconsistent if not conflicting study results for prevalences for these reasons were reported since the mid-1990s, when several Egyptian institutions started surveillance in livestock animals and/or humans targeting *Cryptosporidium* spp. (Abou-Eisha, 1994; Boghdadi, 1996; Abou-Eisha et al., 2000; El-Sibaei et al., 2003; Abdel-Messih et al., 2005; El-Beshbishi et al., 2005; El-Kadi et al., 2006; El-Shazly et al., 2007a; Abdel-Hameed et al., 2008; Shoukry et al., 2009; Antonios et al., 2010; El-Moamly and El-Sweify, 2012; Hassanein et al., 2012; Nora et al., 2012) by using different diagnostic methods based on microscopy, serology and molecular techniques or combinations of these methods.

Amer and co-workers in 2010 started to execute more structured studies with analytic power and using molecular techniques (Amer et al., 2010). Recent estimates for cryptosporidiosis of calves less than 8 weeks age were 30.2 % (Amer et al., 2013) and up to 47% for humans (Abd El Kader et al., 2012). Results of this study are well in the range of these estimates.

5.2 Detected *Cryptosporidium* species

Since molecular analysis became an important tool in the diagnosis and detection of *Cryptosporidium* species, *C. parvum, C. bovis, C. ryanae* and *C. andersoni* were identified as the four major species in cattle worldwide (Fayer et al., 2006; Feng et al., 2007). These species were also identified in Ismailia province. *C. parvum* was the most common species, but *C. ryanae* (11.8%) and mixed infections of *C. parvum* with *C. ryanae* (11.2%) were also present at higher frequencies than seen in most industrialized countries (Amer et al., 2013).

Age-related infection rates followed the known pattern of negative correlation with increasing age. Subclinical infection and lower *Cryptosporidium* prevalence in older animals could be due to several factors, such as an age-related resistance due to maturation of the intestinal mucosa (Harp et al., 1990), species-specific resistance (Fayer et al., 2005)

or partial resistance to other *Cryptosporidium* species. *C. bovis*, *C. ryanae* and *C. andersoni* are truly less pathogenic than *C. parvum,* resulting in low grade infection and lower oocyst output, which in turn reduce the infection pressure among infected animals.

Humans can be infected predominantly with *C. hominis* and *C. parvum* as major species. The distribution of *C. parvum* and *C. hominis* differs in geographic regions. In European countries, both *C. parvum* and *C. hominis* are common in humans; in the Middle East, *C. parvum* is said to be the dominant species in humans. Several studies indicated that humans became earlier and more frequently exposed to *C. parvum* in several 'developing' countries (Steinberg et al., 2004; Teixeira et al., 2007). In the rest of the world, especially in truly developing countries, *C. hominis* is usually the predominant species in humans (Xiao, 2010). Cryptosporidiosis also is a common parasite cause of tourist diarrhea when travelling to more rural areas (Weitzel et al., 2006; Yoder and Beach, 2010).

The investigation showed that infection with *C. hominis* was more prevalent in children than with *C. parvum*. This is in contrast to Xiao (2010) who states a principal higher proportion of *C. parvum* in human infections in the Middle East. Our investigation supports the notion of a unique concentration of *C. hominis* over *C. parvum* cases in children infections in Egypt, if not the wider region, as did another recent study in Egypt using molecular diagnostics (Abd El Kader et al., 2012).

C. parvum and *C. hominis* are thus far associated with most waterborne, food-borne and direct contact-associated (person-to-person and animal-to-person) infections of cryptosporidiosis. Differences in the distribution of the *C. hominis* and *C. parvum* are considered an indication of differences in sources of infection. The predominance of *C. hominis* in a population is considered to be the result of anthroponotic transmission, whereas both anthroponotic and zoonotic transmission are possible routes for *C. parvum* infections (Plutzer and Karanis, 2009).

Surprisingly, rather than occurring particularly in urban Egypt due to anthroponotic transmission, *C. hominis* in this study was more frequently detected in rural areas. Such predominance of anthroponotic species as a cause of human cryptosporidiosis is recorded for some of the developed countries such as Australia, Canada, Japan, USA and some developing countries like Peru, Thailand, and South Africa. In some European countries like France, Belgium and England the relation of *C. parvum/C. hominis* is more balanced and *C. parvum* dominates in the Netherlands and Italy (El-Helalya et al., 2012).

5.3 Detected *C. parvum* subtypes

C. parvum and *C. hominis* in this study were divided into subtypes, based on the sequence of the 60-kGa glycoprotein gene. All four identified *C. parvum* subtypes in this study belonged to the zoonotic subtype families IIa and IId. Two identical subtypes (IIaA15G1R1 and IIdA20G1) were found in both animals and humans. Of these, IIdA20G1 dominated in each case, with 80.2% in animals (IIaA15G1R1: 18.9%) and 50% in humans (IIaA15G1R1: 14.3%). One additional subtype each was detected only in animals but not in humans or vice versa, being IIdA19G1 (0.9%, 1 case) in animals and IIaA15G2R2 (35.7%, 5 cases) in humans.

Calves are commonly infected with subtypes of the IIa subtype family. Totally, subtype IIa was noted to be the most prevailing allele in calves worldwide (Xiao, 2010). Subtypes IIaA15G2R1 and IIa15G1R1 are especially common (Xiao, 2010). In many developed countries in North America (Trotz-Williams et al., 2006) and Europe (Imre et al., 2011), IIaA15G2R1 prevails (Jex and Gasser, 2010; Xiao, 2010). Several other subtypes are more regionally distributed, IIaA16G1R1 is the predominant subtype in Balkan countries (Xiao, 2010).

Other subtype families are more regionally distributed. IId subtypes are occasionally found in calves in European countries, but are especially common in lambs and goat kids (Geurden et al., 2007; Quilez et al., 2008; Imre et al., 2013; Yang et al., 2014) and especially in countries like Spain (Xiao, 2010).

This study confirms that in Egypt, if not the wider region, subtype family IId is the most frequent subtype in animals and humans, with IIdA20G1 being the most prevailing allele. Amer et al. (2010) were the first to identify the occurrence of this IId subtype family in cattle in Egypt. They hypothesize that IId may be due to an accidental infection of cattle from sheep and/or goats, with subsequent adaptation and cycling in cattle and eventual transmission to humans. Meanwhile, in Egypt, IIdA20G1 was also identified to dominate in water buffalo calves (Amer et al., 2013). Because of the large populations of small ruminants in Middle East countries, it seems plausible that in this region zoonotic transmission of *Cryptosporidium* spp. has originated from sheep and is dominated by the IIdA20G1 subtype. IIdA20G1, however, also has been identified in cattle to dominate in regions without a sheep/goat background. Mi et al. (2013) reported that IIdA20G1 was identified in China as an only subtype in dairy calves previously.

For Egypt, thus a unique, distinct picture arises: cryptosporidiosis in livestock (cattle and buffalo) is characterized by the dominance of the allele family IId and the common occurrence of non-*C. parvum* species (Amer et al., 2013).

No subtypes were identified for *C. hominis*, probably due to low numbers of oocysts and lower sensitivity of the Gp60 specific PCR compared to the 18S rDNA specific PCR. Within the 38.5% portion of human infections due to *C. parvum,* subtype IIdA20G1, as in the animals, did dominate (50%), but followed by IIaA15G2R1 rather than IIaA15G1R1 as in animals.

C. parvum subtype families IId and IIa, responsible for zoonotic transmission, therefore, were found in both humans and ruminants, with IIdA20G1 by far dominating, followed by noticeable lower frequencies and in particular different mixes of IIa subfamily alleles.

The study area picture thus agrees with other Middle Eastern countries, where nearly all C. *parvum* IId subtypes from human isolates were identified to be IIdA20G1 (Sulaiman et al., 2005; Amer et al., 2010; Hijjawi et al., 2010). Children in Kuwait City were almost exclusively infected with almost equal numbers of IIa (predominantly IIaA15G2R1) and IId (predominantly IIdA20G1) subtypes, although they had little contact with farm animals, the city uses desalinized sea water as drinking water, and the transmission appeared to be anthroponotic in origin (Sulaiman et al., 2005). IId subtypes are also common in children in Saudi Arabia (Al-Brikan et al., 2008). In Ethiopia, in contrast, three IIa subtypes, mainly IIaA15G2R2, but no IId were found in human cryptosporidiosis cases (Adamu et al., 2010).

All *C. parvum* of the subtype family IIa detected in cattle and buffalo samples and in two human cases did belong to the IIaA15G1R1 subtype as previously reported in several regions (Soba and Logar, 2008; Kvac et al., 2011). IIaA15G1R1 within the IIa subtype family, however, did not prevail in humans, where rather IIaA15G2R1 occurred. IIaA15G2R1 was previously found to be the most common genotype in Malaysian HIV patients (Iqbal et al., 2012), identified from a patient in France (Certad et al., 2012), in beef calves in France (Rieux et al., 2014), in an extensive cattle farming region of northern Tunisia (Rahmouni et al., 2014) and in children from Mexico (Valenzuela et al., 2014).

A predominance of IIaA15G2R1 in the human IIa subtypes traditionally is associated with livestock animals, but the biological and medicinal differences between *Cryptosporidium* subtype families and particularly individual subtypes are not clear.

5.4 Transmission dynamics, transmission pathways

Differences in infection sources could be an important factor for differences in *Cryptosporidium* transmission between different countries and areas within countries. The distribution of *Cryptosporidium* cases with a dominance of *C. hominis* in Egypt in children in this study is in agreement with African and Asian countries (Tumwine et al., 2003). Anthroponotic transmission by *C. hominis* as a single species seen in about half the cases of diarrheic children seems to be the most important pathway of infection. Zoonotic transmission from animals and water on the other hand is not only caused by *C. parvum* but by at least 3 further *Cryptosporidium* species of cattle.

Where *C. parvum* in this study was the only zoonotic species of animals, the IId subtype was most common. The dominance of the IId allele also in the human isolates points to the predominance of zoonotic transmission by *C. parvum* with its prevailing IId allele.

Heterogeneity among *Cryptosporidium* species in livestock animals points to different species of vertebrates and suggests that from these a series of host-adapted genotypes/strains/species have evolved and circulate.

The predominance of single subtypes or mixes of subtypes dominating in herds gives hints to likely herd management systems. Studies from areas with closed herd management (few animal movements between herds) have shown a high number of GP60 subtypes in the calf population, but one isolated, distinct subtype population within any herd (Misic and Abe, 2007; Brook et al., 2009). In contrast, only a few subtypes were identified in areas with higher exchange rates between herds, but with several subtypes being present in a herd (Brook et al., 2009). Thus, subtyping of calf samples have shown the same within-herd pattern, with more mixed subtype infections per herd in Turkey, where herds more frequently mix, than in Israel, where closed herds are more common (Tanriverdi et al., 2006). The number of detected *Cryptosporidium* species and the subtype species mixes within herds, with IId dominance, in this study also points to extensive, open keeping of herds with frequent exchanges between herds.

The smallholder system in the study area does not isolate single herds, where animals during the day rather are left to themselves and have contact with animals from other holdings.

5.5 Risk factors and control options

At the herd and the human sample levels, four investigated factors, as in other studies, were associated with *Cryptosporidium* prevalence: drinking water, risk age groups of animals and humans, and contact of humans with animals.

Drinking water, particularly contaminated surface/underground and canal water, was identified as a major risk factor for animals. *C. parvum* was about eight times more frequently detected in animals watered with underground/canal than with tap water. The kind of water also was identified a risk factor for cryptosporidial infection in children, where *Cryptosporidium* prevalence was about 1.7 times higher when children had drunk underground rather than tap water.

It is noted that livestock animals were the major source of *Cryptosporidium* oocyst contamination of surface water. Sewage is subjected to minimal treatment and effluent is discharged into canals, lakes and seas. Contamination of underground water or shallow wells often happens in a high quantity (Antonios et al., 2001; Khalifa et al., 2001) by runoff surface water, particularly in the Ismailia province. Rayan et al. (2009) confirmed 3% oocyst prevalence in Ismailia even in tap water. Tap water from water treatment plants which use rapid sand filters and sequentially add chlorine-based disinfectants thus is apparently not entirely free from *Cryptosporidium* although of better hygienic quality, particularly in regards to *C. parvum* (Abou-Eisha et al., 2000).

Age was a major risk factor in animals which led to a significant 2.7-fold decrease from young to adult cattle and buffaloes (Singh et al., 2006; Nasir et al., 2009). In children a higher prevalence of *Cryptosporidium* also was observed in the younger age group (1.5- 6 years old, when they enter primary school). The principal age pattern observed (mostly children younger than 5 years infected) was similar to other developing countries (Bhattacharya et al., 1997; Newman et al., 1999; Bern et al., 2000; Gatei et al., 2006). As in other studies, contact with animals was the third risk factor identified. Diarrheic children that had a history of contact with ruminants had a significantly higher *Cryptosporidium* prevalence than children with no history of contact with animals (Hale et al., 2012; Wegayehu et al., 2013; Adamu et al., 2014).

Prevention and control of cryptosporidiosis require continued efforts to interrupt the transmission of *Cryptosporidium* through water, food, and contact with infected persons and animals. Hygiene measures at any setting are essential. Of particular importance is

continuous improvement and monitoring of respective run-off water sources and non-treated water.

Veterinarians could play a role in implementing hygiene measures to be adopted and regulated in the farm animal environment, but access of animals to surface and canal water realistically cannot be restricted. Prevention and control programs will have to be multi-faceted. Land-use regulations, economic incentives, and educational efforts towards behavioral change may be necessary for the implementation and the success of long-term strategies. Routinely testing stool specimens of diarrheic patients for cryptosporidiosis in hospitals is an option that can be implemented short term.

5.6 Conclusions

1. High prevalence of *Cryptosporidium* indicates that this protozoan parasite is ubiquitous in animals and humans in the Ismailia province of Egypt.
2. All *Cryptosporidium* previously reported as common in livestock animals (*C. parvum, C. ryanae, C. bovis* and *C. andersoni*) and in children (*C. hominis, C. parvum,* and *C. bovis*) are present in the province. *C. parvum* dominates in animals, but *C. ryanae*. *C. bovis* and *C. andersoni* are also present in non-negligible numbers.
3. Inter-species diversity between human and animal isolates points to two clearly separated transmission cycles. Anthroponotic infection is through *C. hominis* and zoonotic infection is mainly due to *C. parvum* through contact with contaminated water and animals.
4. Prevailing risk factors favor widespread and dynamic scenarios of zoonotic and particularly anthroponotic transmission. Anthroponotic transmission unusually is occurring not urban but rather rural in aggregations of people along the irrigation waterways.
5. Rather than IIa, the IId subfamily with IIdA20G1 as most frequent subtype dominates zoonotic transmission with *C. parvum*. Farm animals interact with the waterways and contaminate surface and runoff waters.
6. For Egypt, thus a unique, distinct picture arises: cryptosporidiosis in livestock (cattle and buffalo) is characterized by the dominance of the allele family IId and by the common occurrence of non-*C. parvum* species.
7. Animal infections with non-*C. parvum* species and exchange of these species between animals are frequent and favored by the essentially extensive husbandry system, enabling frequent contacts of animals of different herds.

8. Likely control efforts have to aim at interrupting the zoonotic pathways as well as the anthroponotic infection chains. The task is multidimensional, multifaceted. Realistic is to initiate short-term routine testing of stool specimens of diarrheic patients for *Cryptosporidium* in hospitals.

6 Summary

Cryptosporidiosis is an underestimated problem in livestock animals and humans in Egypt. To provide a valid estimate of the frequencies and distributions of *Cryptosporidium* species and their subtypes in both animals and humans, a sample of smallholder cattle/buffalo herds in the districts of Ismailia province and of diarrheic children attending the province's district hospitals was investigated. A total of 804 animal samples from 191 herds and 165 human samples were investigated by the Copro-antigen *RIDA®QUICK* test, followed by PCRs (animals: all *RIDA®QUICK* test positive + 10% negative) targeting the 18S rDNA and Gp60 genes and PCR-RFLP assays for amplification and differentiation of *Cryptosporidium* spp. Detailed analyses were carried out for prevalence, distribution, risk factors, and infection dynamics of cryptosporidiosis and involved *Cryptosporidium* species and subtypes. Results of molecular diagnostic methods were co-analyzed with epidemiological information. Cryptosporidiosis was found to be common in Ismailia province; within herd prevalence was 73.3%, individual animal prevalence 32.2% and prevalence in diarrheic children 49.1%.

The pattern of cryptosporidiosis is unique, distinct and multilayered in the province. Two independent transmission cycles exist. Anthroponotic transmission is due to *C. hominis*. Infection hotspots, however, are not urban but rather rural areas along the province's major irrigation canal. The zoonotic scenario is characterized by 5 *Cryptosporidium* species involved, with *C. parvum* dominating, and by 4 *C. parvum* subtypes, not all identical in animals and humans. The surprising dominance of the IId allele family in animals and humans points to a likely adaptation of a typical subtype of small ruminants. An endemic cryptosporidiosis situation in livestock is likely, maintained by the predominant smallholder husbandry system, where the frequent exchange between herds reshapes the existence of several *Cryptosporidium* species and subtypes in animals.

This animal scenario is principally mirrored in zoonotic cryptosporidiosis (*C. parvum*) of humans. Development of control strategies should take notice of the special features identified in animal and human cryptosporidiosis. Strategies will have to be long-term, include hygienic measures at any setting and behavioral change of people, both rural and urban. The introduction of routine testing of stool specimens of diarrheal patients is a realistic short term option.

7 Zusammenfassung

Epidemiologische Untersuchungen zur Public Health-Bedeutung von *Kryptosporidien* von Nutztieren und Menschen in der Ismailia-Kanal Zone Ägyptens

Kryptosporidiose landwirtschaftlicher Nutztiere und der menschlichen Bevölkerung ist ein unterschätztes Problem in Ägypten. Zur Erstellung valider Schätzungen von Häufigkeiten und Verteilungen von Kr*yptosporidien*-Spezies und ihrer Genotypen sowohl in Nutztieren als auch Menschen der Ismailia Provinz Ägyptens wurden Stichproben von Herden ägyptischer Rinder und Büffel des vorherrschenden kleinstbäuerlichen Haltungssystems aus den Distrikten der Provinz und von Kindern mit Durchfall, die stationäre Patienten eines der Distrikthospitäler waren, untersucht. Insgesamt wurden 804 Proben von Tieren aus 191 Herden und 165 Humanproben mit dem Copro-antigen *RIDA®QUICK* Test, gefolgt von auf die 188 rDNA und das Gp60-Gen abzielende PCRs (Tiere: alle *RIDA®QUICK* Test positiv + 10% negativ) und PCR-RFLP-Assays zur Amplifizierung und Differenzierung von *Kryptosporidium* spp. untersucht. Detaillierte Analysen wurden zur Prävalenz, Verteilung, Risikofaktoren und Infektionsdynamik von Kryptosporidiose und beteiligter *Kryptosporidium*-Spezies und Subtypen durchgeführt. Ergebnisse der molekularen Diagnostik wurden zusammen mit epidemiologischen Informationen analysiert. Kryptosporidiose war häufig in der Ismailia-Provinz, die Herdenprävalenz lag bei 73,3%, die Einzeltierprävalenz bei 32.2% und die Prävalenz bei Kindern mit Durchfall bei 49.1%. Das Muster der Kryptosporidiose in der Provinz ist einzigartig, unterschiedlich und mehrschichtig im Vergleich zu anderen Regionen und Ländern. Zwei unabhängige Infektionswege bestehen. *C. hominis* ist verantwortlich für Mensch-zu-Mensch-Übertragungen. Infektions-Hotspots liegen jedoch nicht in urbanen, sondern ländlichen Gegenden entlang des Hauptbewässerungskanals der Provinz. Am zoonotischen Szenarium sind fünf *Kryptosporidium*-Spezies, dominiert von *C. parvum*, und 4 *C. parvum*-Subtypen, zum Teil unterschiedlich in Tieren und Menschen, beteiligt. Die überraschende Dominanz der IId Allel-Familie in Tieren und Menschen deutet auf eine mögliche Adaptation eines typischen Subtyps kleiner Wiederkäuer hin. Eine endemische Kryptosporidiose-Situation in landwirtschaftlichen Nutztieren ist naheliegend, die durch das vorherrschende Kleinstbauern-System unterstützt wird, das, durch unregulierte Kontakte von Herden, Umformung und Austausch verschiedener *Kryptosporidium*-Spezies und Subtypen in Tieren fördert. Dieses Tier-Szenarium spiegelt sich grundsätzlich in der zoonotischen Kryptosporidiose durch *C. parvum* auch bei Menschen wieder. Die

Entwicklung von Kontrollstrategien in Ägypten sollte die speziellen Merkmale der Kryptosporidiose in Tieren und Menschen berücksichtigen. Strategien müssen langfristig angelegt sein und hygienische Maßnahmen auf allen Ebenen sowie Verhaltensänderungen sowohl der ländlichen wie städtischen Bevölkerungen einschliessen. Die Einführung von Routineuntersuchungen von Stuhlproben von Patienten mit Durchfall ist eine realistische, kurzfristig wirksame Option.

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9 List of published articles

9.1 Publications with Peer-Review process:

1. **Helmy YA.**, Klotz C., Wilking H., Krücken J., Nöckler J, Von Samson-Himmelstjerna G., Zessin KH. and Aebischer T. (2014): Epidemiology of *Giardia duodenalis* infection in ruminant livestock and children in the Ismailia province of Egypt: Insights by genetic characterization. Parasites & Vectors 7: 321-332.
2. **Helmy YA.**, Von Samson-Himmelstjerna G., Nöckler K., and Zessin KH. (2014): Frequencies and spatial distributions of *Cryptosporidium* of livestock animals and children in the Ismailia province of Egypt. Epidemiology and Infection 1: 1-11.
3. **Helmy YA.**, Krücken J., Nöckler K., Von Samson-Himmelstjerna G. and Zessin KH. (2013): Molecular epidemiology of *Cryptosporidium* in livestock animals and humans in the Ismailia province of Egypt. Veterinary Parasitology 193 (1-3): 15-24.
4. **Helmy YA.**, Krücken J., Nöckler K., Von Samson-Himmelstjerna G. and Zessin KH. (2013): Comparison between two commercially available serological tests and polymerase chain reaction in the diagnosis of *Cryptosporidium* in animals and diarrheic children. Parasitology Research 113(1): 211-216.

9.2 List of Abstracts in Academic and Scientific Conferences

1. **Yosra A. Helmy**, Jürgen Krücken, Georg von Samson-Himmelstjerna, Karl-H. Zessin and Hafez M. Hafez (2014). Molecular detection and species identification of *Cryptosporidium* spp. in chicken and turkey feces in Germany. The 10th "Hafez" International Symposium on Turkey Diseases. 05-07 June, Berlin, Germany.
2. **Yosra A. Helmy**, Jürgen Krücken, Christian Klotz, Karsten Nöckler, Toni Aebischer, Georg von Samson-Himmelstjerna and Karl-H. Zessin (2014). Epidemiology and molecular genotyping of *Cryptosporidium* and *Giardia* in cattle, buffalo and humans in the Ismailia province of Egypt. The 5th International *Giardia & Cryptosporidium* Conference. 27- 31 May, Uppsala, Sweden.
3. **Yosra A. Helmy**, Jürgen Krücken, Georg von Samson-Himmelstjerna, Karl-H. Zessin and Hafez M. Hafez (2014). Molecular characterization and genotyping of *Cryptosporidium* spp. in chicken and turkeys in Germany. The 5th International *Giardia & Cryptosporidium* Conference. 27- 31 May, Uppsala, Sweden.
4. **Yosra A. Helmy,** Christian Klotz, Jürgen Krücken , Karsten Nöckler, Georg von Samson-Himmelstjerna, Karl-H. Zessin and Toni Aebischer (2014). The public health significance of *Giardia duodenalis* in livestock and children in the Ismailia canal zone

of Egypt: Epidemiology and molecular analysis. The 24th European Congress of Clinical Microbiology and Infectious Diseases conference. 9-13 May, Barcelona, Spain.

5. **Yosra A. Helmy**, Jürgen Krücken , Karsten Nöckler, Georg von Samson-Himmelstjerna and Karl-H. Zessin (2013). Evaluation of two copro- antigen tests and comparison with polymerase chain reaction in the diagnosis of *Cryptosporidium* in animals and humans, The 2nd International Meeting on Apicomplexean Parasites in Farm Animals. 31 October - 3 November, Kusadasi, Turkey.
6. **Yosra A. Helmy**, Toni Aebischer, Jürgen Krücken, Karsten Nöckler, Georg von Samson-Himmelstjerna Christian Klotz and Karl-H. Zessin (2013). Multilocus genotyping and zoonotic importance of *Giardia duodenalis* of animals and humans of the Ismailia province of Egypt. National Symposium on Zoonoses Research. 19-20 September, Berlin, Germany.
7. **Yosra A. Helmy**, Jürgen Krücken, Georg von Samson-Himmelstjerna, Karl-H. Zessin, Dörte Lüschow and Hafez M. Hafez (2013). Molecular detection and identification of *Cryptosporidium* spp. in poultry in Germany. The 18th Congress of the World Veterinary Poultry Association. 19-23 August, Nantes, France.
8. **Yosra A. Helmy**, Christian Klotz, Toni Aebischer, Jürgen Krücken, Karsten Nöckler, Georg von Samson-Himmelstjerna and Karl-H. Zessin (2013). Multilocus genotyping of *Giardia duodenalis* in cattle, buffalo and human from Ismailia province of Egypt. The 23rd European Congress of Clinical Microbiology and Infectious Diseases. 27- 29 April, Berlin, Germany.
9. **Yosra A. Helmy**, Jürgen Krücken, Karsten Nöckler, Georg von Samson-Himmelstjerna and Karl-H. Zessin (2012). Cryptosporidiosis in cattle, buffalo and humans in the Ismailia province of Egypt: Epidemiology and molecular analysis. The 1st Apicomplexian Conference. 25- 28 October, Lisbon, Portugal.
10. **Yosra A. Helmy**, Jürgen Krücken, Karsten Nöckler, Georg von Samson-Himmelstjerna and Karl-H. Zessin (2012). Prevalence of *Cryptosporidium* in animals and humans in the Ismailia province in Egypt. The 25th Annual Meeting of the German Society for Parasitology. 14- 17 March, Heidelberg, Germany.

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Selbständigkeitserklärung:

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbständig angefertigt habe. Ich versichere, dass ich ausschliesslich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

Yosra Ahmed Helmy Abdelsamad Mohamed

Berlin, den 04.09.2014