JIHOČESKÁ UNIVERZITA V ČESKÝCH BUDĚJOVICÍCH

ZEMĚDĚLSKÁ FAKULTA

Kryptosporidie a kryptosporidióza ptáků

Cryptosporidium and cryptosporidiosis in birds

Disertační práce

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Školitel: **prof. Ing. Martin Kváč, Ph.D.**

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Disertační práce

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Předkládám tímto k posouzení a obhajobě disertační práci zpracovanou na závěr doktorského studia na Zemědělské fakultě Jihočeské univerzity v Českých Budějovicích. Prohlašuji tímto, že jsem práci vypracovala samostatně, s použitím odborné literatury a dostupných zdrojů uvedených v seznamu, jenž je součástí této práce. Dále prohlašuji, že v souladu s § 47b zákona č. 111/1998 Sb. v platném znění, souhlasím se zveřejněním své disertační práce, a to v úpravě vzniklé vypuštěním vyznačených částí archivovaných Zemědělskou fakultou, elektronickou cestou ve veřejně přístupné sekci databáze STAG, provozované Jihočeskou univerzitou v Českých Budějovicích na jejích internetových stránkách.

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V Českých Budějovicích dne 18. listopadu 2021

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Ing. Nikola Holubová

Seznam impaktovaných publikací

Disertační práce vychází z těchto publikací:

I. Chelladurai J.J., Clark M.E., Kváč M., Holubová N., Khan E., Stenger B.L., Giddings C.W., McEvoy J. 2016: *Cryptosporidium galli* and novel *Cryptosporidium* avian genotype VI in North American red-winged blackbirds (*Agelaius phoeniceus*). Parasitology Research 115: 1901–1906.

II. Holubová N., Sak B., Horčičková M., Hlásková L., Květoňová D., Menchaca S., McEvoy J., Kváč M. 2016: *Cryptosporidium avium* n. sp. (Apicomplexa: Cryptosporidiidae) in birds. Parasitology Research 115: 2243–2251.

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Prohlášení spoluautorů

Všichni níže uvedení spoluautoři prohlásili, že Ing. Nikola Holubová se podstatným způsobem podílela na přípravě a publikování rukopisů, ve kterých není prvním autorem, přiložených k této disertační práci. Souhlas jednotlivých spoluautorů byl elektronicky zaslán školiteli doktorandky prof. Ing. Martinu Kváčovi, Ph.D., a byl ověřen předsedou OR DSP Zoohygiena a prevence chorob hospodářských zvířat Zemědělské fakulty Jihočeské univerzity v Českých Budějovicích prof. Ing. Janem Trávníčkem, CSc.

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Další impaktované publikace, na kterých jsem spolupracovala a které se přímo netýkají tématu disertační práce (nejsou součástí práce):

VIII. Kváč M., **Hromadová N.**, Květoňová D., Sak B. 2011: Molecular characterization of *Cryptosporidium* spp. in pre-weaned dairy calves in the Czech Republic: absence of *C. ryanae* and management-associated distribution of *C. andersoni*, *C. bovis* and *C. parvum* subtypes. Veterinary Parasitology 177: 378–382.

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GAČR 18-12364S – Kryptosporidie ptáků: doplnění chybějících znalostí u neoprávněně opomíjené skupiny hostitelů (2018–2020; řešitel: prof. Ing. Martin Kváč, Ph.D.).

MSMT LTAUSA17165 – Diverzita a koevoluce kryptosporidií hlodavců: propojení studia genetické variability a biologie parazitů (2017–2020; řešitel: prof. Ing. Martin Kváč, Ph.D.).

GAJU 082/2017/Z – Prevalence, diverzita a biologie kryptosporidií parazitujících u vrubozobých (2017; řešitelka: Ing. Nikola Holubová).

GAJU 017/2018/Z – Kryptosporidie volně žijících ptáků: diverzita, biologie a možnost přenosu na hospodářská zvířata (2018; řešitelka: Ing. Nikola Holubová).

GAJU 040/2019/Z – Ptačí vejce jako inkubátor pro pomnožení parazitů rodu *Cryptosporidium*: možnost překonání hostitelské a mezidruhové specifity (2019; řešitelka: Ing. Nikola Holubová).

GAJU 016/2020/Z – Synantropní hlodavci: hostitelé zoonotických a hostitelsky specifických kryptosporidií (2020; řešitelka: RNDr. Jana Ježková, spoluřešitelka: Ing. Nikola Holubová).

GAJU 007/2021/Z – Zdivočelí holubi jako zdroj zoonotických parazitů rodu *Cryptosporidium*, *Encephalitozoon* a *Enterocytozoon* (2021; řešitelka: Ing. Nikola Holubová).

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Předložená disertační práce zahrnuje výsledky týkající se kryptosporidií a kryptosporidiózy ptáků, které byly získány na základě multidisciplinárního přístupu zahrnujícího molekulární biologii, parazitologii, zoologii, histologii a experimentální infekce a byly publikovány v sedmi vědeckých publikacích v impaktovaných časopisech.

Anotace

Cryptosporidium (Apicomplexa) je rod jednobuněčných parazitů infikujících gastrointestinální, respirační a urogenitální trakt většiny obratlovců včetně člověka a způsobujících onemocnění kryptosporidiózu. Podle Světové zdravotnické organizace je kryptosporidióza celosvětově se vyskytujícím průjmovým onemocněním, které postihuje miliony osob, je druhou nejčastější příčinou úmrtí kojenců v rozvojových zemích a je označována za novou příčinu nemocnosti a úmrtnosti na celém světě. Kryptosporidie také způsobují závažné onemocnění u hospodářských zvířat, kde vyvolávají časté průjmy a způsobují značné ekonomické ztráty, zejména u mladých jedinců. Ačkoli jsou kryptosporidie intenzivně zkoumány již více než 35 let, výzkum se do značné míry zaměřuje na kryptosporidie u lidí, hospodářských zvířat a jiných savců, přičemž kryptosporidiím ptáků byla dosud věnována poměrně malá pozornost. V současné době je popsáno 49 platných druhů kryptosporidií parazitujících u širokého spektra hostitelů. Kromě platně popsaných druhů byly popsány desítky genotypů, o kterých není dostatek údajů, aby bylo možné je označit za samostatné druhy. Z těchto druhů a genotypů je šest druhů, z nichž tři byly popsány v rámci této práce, a 20 genotypů specifických pro ptáky. Tato disertační práce výrazně rozšiřuje znalosti o kryptosporidiích specifických pro ptáky, zaměřuje se na jejich výskyt a diverzitu v rámci 25 řádů a 176 čeledí ptáků, morfologii vývojových stádií, hostitelskou a orgánovou specifitu, patogenitu a přenos.

Annotation

Cryptosporidium is a genus of single-cell protist parasites that infect gastrointestinal, respiratory and/or urogenital tract of most vertebrates, including humans, and it causes the disease cryptosporidiosis. According to the World Health Organisation, cryptosporidiosis is a global diarrhoeal disease affecting millions of individuals; it is the second most common cause of infantile death in developing countries and is has been identified as an emerging cause of morbidity and mortality worldwide. The disease is also severe in livestock, causing profuse diarrhoea and considerable economic losses in farmed young animals. Although *Cryptosporidium* has been under intensive investigation for more than 35 years, research has been heavily biased towards *Cryptosporidium* in humans, livestock, and other mammals, with comparatively little attention paid to *Cryptosporidium* in birds. Currently, there are 49 described valid *Cryptosporidium* species infecting a wide spectrum of animals. In addition to the validly described species, dozens of genotypes have been described that lack sufficient data to justify a species designation. Out of these, six species, three were described within this thesis, and 20 genotypes has been reported to be bird specific. The thesis greatly expands the overview of bird-derived *Cryptosporidium*, focusing on its prevalence and diversity across 25 orders and 176 families within the class Aves, morphology of developmental stages, host- and organ specificity, pathogenicity and transmission.

Souhrn

Předmětem této disertační práce je studium výskytu a diverzity kryptosporidií infikujících ptáky. Během studia bylo získáno a vyšetřeno pomocí mikroskopických a molekulárních metod 4740 vzorků trusu ptáků z 25 řádů a 176 rodů z 9 zemí, konkrétně 2928 vzorků z České republiky, 301 ze Slovenska, 732 z Chorvatska, 25 z Polska, 90 z Jihoafrické republiky, 238 z Nového Zélandu, 11 ze Spojených arabských emirátů, 345 z Alžírska a 70 z USA. Specifická DNA kryptosporidií byla detekována na lokusech genů kódujících malou podjednotku rRNA (SSU), aktin, heat shock protein (HSP70), thrombospondin-related adhesive protein (TRAP-C1), *Cryptosporidium* oocyst wall protein (COWP) a 60 kDa glykoprotein (gp60) v 255 případech (5,4 %). Fylogenetické analýzy prokázaly přítomnost 11 druhů a 6 genotypů kryptosporidií. Nejčastěji byly detekovány druhy *C. baileyi* (65/4740; 1,4 %), *C. proventriculi* (35/4740; 0,7 %) a *Cryptosporidium* sp. goose genotyp Id (33/4740; 0,7 %). Naopak nejméně často byl detekován *Cryptosporidium* sp. avian genotyp I (2/4740; 0,04 %). Na základě fylogenetických a experimentálních studií byl popsán jeden nový genotyp - *Cryptosporidium* avian genotyp VI z vlhovce červenokřídlého a tři nové druhy - *C. avium* z kakarikiho rudočelého, *C. proventriculi* z papouška koňžského a *C. ornithophilus* z pštrosa dvouprstého. Velikost oocyst všech nově popsaných druhů se od sebe lišila. Oocysty *C. avium* měřily 6,3 × 4,9 μm, *C. proventriculi* 7,4 × 5,7 μm a *C. ornithophilus* 6,1 × 5,2 μm. Studium tkáňové specifity prokázalo vývoj *C. avium* v tenkém a slepém střevě, ale také ledvinách a močovodech. Vývojová stádia *C. proventriculi* byla nalezena v proventrikulu a ventrikulu. Druh *C. ornithophilus* infikoval slepé a tlusté střevo spolu s Fabriciovou burzou. *Cryptosporidium avium* získané z papoušků, bylo infekční pro kur domácí z řádu hrabavých, kachnu domácí patřící do řádu vrubozobých, ale překvapivě neinfekční pro bažanta obecného. Druh *C. proventriculi* je infekční pro korelu chocholatou z řádu papoušků, nikoliv však andulku vlnkovanou, která také patří mezi papoušky. Kur domácí byl v experimentálních podmínkách vnímavý k infekci *C. ornithophilus*. Ani jeden výše jmenovaný druh kryptosporidií nebyl infekční pro laboratorní myši kmene SCID a BALB/c. Výsledky našich studií výrazným způsobem rozšířily naše znalosti o věkové specifitě a průběhu infekce různých druhů a genotypů kryptosporidií parazitujících u ptáků. *Cryptosporidium*

avium patří mezi druhy kryptosporidií, u kterých byl prokázán přenos parazita na hostitele u všech věkových kategorií vybraných skupin ptáků. U *Cryptosporidium ornithophilus* jsme na základě námi provedených experimentů zjistili kratší prepatentní periodu u mladších jedinců než u dospělých, kteří mohou být k infekci méně citliví z důvodů již prodělané kryptosporidiové infekce. Během našich experimentů jsme nezaznamenali žádné klinické příznaky ptáků infikovaných druhy *C. avium*, *C. proventriculi* a *C. ornothophilus*. V neposlední řadě byla tato disertační práce rozšířena o výzkum zaměřený na kultivaci kryptosporidií pomocí kuřecích embryí. Byla úspěšně zpracována a opakovaně ověřena metodika pro úspěšnou infekci embryí druhy *C. baileyi* a *C. parvum*.

Summary

The subject of this thesis is the study of the occurrence and diversity of cryptosporidia infecting birds. During the study, 4,740 bird faecal samples from 25 orders and 176 genera from 9 countries, namely 2,928 samples from the Czech Republic, 301 from Slovakia, 732 from Croatia, 25 from Poland, 90 from South Africa, 238 from New Zealand, 11 from the United Arab Emirates, 345 from Algeria and 70 from the USA, were obtained and examined using microscopic and molecular methods. *Cryptosporidium*-specific DNA was detected at loci of genes encoding small subunit rRNA (SSU), actin, heat shock protein (HSP70), thrombospondin-related adhesive protein (TRAP-C1), *Cryptosporidium* oocyst wall protein (COWP) and 60 kDa glycoprotein (gp60) in 255 cases (5.4 %). Phylogenetic analyses revealed the presence of 11 species and 6 genotypes of *Cryptosporidium*. The most frequently detected species were *C. baileyi* (65/4,740; 1.4 %), *C. proventriculi* (35/4,740; 0.7%) and *Cryptosporidium* sp. goose genotype Id (33/4,740; 0.7%). In contrast, *Cryptosporidium* sp. avian genotype I was the least frequently detected (2/4,740; 0.04%). Based on phylogenetic and experimental studies, one new genotype, *Cryptosporidium* avian genotype VI, was described from the red-winged blackbird, and three new species, *C. avium* from the red-fronted parakeet, *C. proventriculi* from the red-fronted parrot and *C. ornithophilus* from the ostrich. The oocyst size of all newly described species differed from each other. Oocysts of *C. avium* measured 6.3 × 4.9 μm, *C. proventriculi* 7.4 × 5.7 μm and *C. ornithophilus* 6.1 × 5.2 μm. Tissue specificity studies showed the development of *C. avium* in the small intestine and caecum but also in the kidney and ureters. Developmental stages of *C. proventriculi* were found in the proventriculus and ventriculus. *Cryptosporidium ornithophilus* infected the caecum and colon together with bursae of Fabricius. *Cryptosporidium avium*, obtained from parrots, was infectious to the domestic chicken belonging to the order Galliformes, the domestic duck belonging to the order Anseriformes, but surprisingly non-infectious to the common pheasant. The species *C. proventriculi* was infectious to the cockatiel of the order Psittaciformes, but not to the budgerigar, which also belongs to the order Psittaciformes. The domestic chicken was susceptible to infection by *C. ornithophilus* under experimental conditions. Neither of the above-mentioned *Cryptosporidium* species was infective to laboratory mice of

the SCID and BALB/c strains. The results of our studies have greatly increased our knowledge of the age specificity and infection course of different species and genotypes of cryptosporidia parasitizing birds. *Cryptosporidium avium* is one of the cryptosporidial species that have been shown to be infectious to the host at all ages in selected groups of birds. In case of *Cryptosporidium ornithophilus*, based on our experiments, we found a shorter prepatent period in younger individuals than in adults, which may be less susceptible to infection because of previous cryptosporidial infection. During our experiments, we did not observe any clinical signs in birds infected with *C. avium*, *C. proventriculi* and *C. ornothophilus* species. Finally, this thesis has been extended to include research on *Cryptosporidium* cultivation using chicken embryos. A methodology for successful infection of embryos with *C. baileyi* and *C. parvum* species was successfully developed and repeatedly validated.

OBSAH

1. ÚVOD

Miliony let trvající vývoj ptáků směřoval zřejmě k jednomu cíli, a to k ovládnutí vzdušného prostoru. Postupem času určité skupiny ptáků z rozličných důvodů schopnost létat ztratily. Někteří ptáci dorostli takových rozměrů, že jejich obrovská těla by svaly křídel nemohly unést (pštros dvouprstý (*Struthio camelus*)), mořští ptáci se specializovali na lov potravy pod hladinou a jejich tělo se zdokonalovalo v plavání, zatímco na souši si vystačili jen s pomalou a kolébavou chůzí a létat vůbec nepotřebovali (tučňáci – Sphenisciformes). Další ptáci se schopnosti létat "dobrovolně" vzdali (kiviové (*Apterygiformes*), papoušek zemní (*Pezoporus wallicus*) a kakapo soví (*Strigops habroptila*), protože ji v životě nepotřebovali a neuplatňovali. Vývoj některých ptáků probíhal na jednom místě, často na izolovaných ostrovech nebo v nedostupných oblastech, poskytujících dostatek potravy a spolu s nimi se vyvíjeli i jejich paraziti. Naopak jiní ptáci díky své schopnosti migrovat na velké vzdálenosti mohli přenášet různé patogeny z jedné oblasti do druhé a představovat tak potenciální zdroj infekce pro další ptačí druhy, ale i člověka a jím chovaná zvířata (Ryan et al. 2014, Nakamura et Meireles 2015).

Paraziti rodu *Cryptosporidium* jsou nejčastější původci parazitárních onemocnění volně žijících, v zajetí a hospodářsky chovaných ptáků (O'donoghue 1995, Sréter et Varga 2000). Vzhledem k omezenému množství studií provedených na ptačích hostitelích, zůstává mnoho otázek nezodpovězených. Mezi základní otázky patří **i)** je druhová diverzita ptačích kryptosporidií obdobná té, kterou známe u kryptosporidií savců, **ii**) jsou ptačí kryptosporidie hostitelsky, věkově a tkáňově specifické, **iii)** jaká je patogenita a průběh infekce ptačích kryptosporidií nebo **iv)** mohou být ptáci hostitelé savčích kryptosporidií?

Přestože počet druhů ptáků výrazně převyšuje počet zástupců třídy savců, u nichž byla popsána velká diverzita kryptosporidií, bylo dosud popsáno pouze 6 platných druhů ptačích kryptosporidií, z nichž 3 (tučně zvýrazněno) byly popsány v rámci této práce: *Cryptosporidium meleagridis* (Slavin 1955), *Cryptosporidium baileyi* (Current et al. 1986), *Cryptosporidium galli* (Pavlásek 1999, Ryan et al. 2003b), ***Cryptosporidium avium*** (Holubová et al. 2016), ***Cryptosporidium proventriculi*** (Holubová et al. 2019) a ***Cryptosporidium ornithophilus*** (Holubová et al. 2020) a 20 genotypů, o kterých nemáme dostatek údajů, aby bylo možné je označit za samostatné druhy. Tato disertační práce výrazně rozšiřuje přehled o kryptosporidiích

specifických pro ptáky, zaměřuje se na jejich výskyt a diverzitu v rámci 25 řádů a 176 čeledí, morfologii vývojových stadií, hostitelskou a orgánovou specifitu, patogenitu a přenos infekce.

2. CÍLE PRÁCE

Cílem této práce je studovat výskyt, diverzitu a biologii ptačích kryptosporidií u vybraných skupin ptáků.

Následující konkrétní dílčí cíle představují jednotlivé kroky výzkumu nezbytné pro dosažení hlavního cíle:

- Vyhodnotit výskyt a prevalenci kryptosporidiových infekcí u volně žijících ptáků, u ptáků ze zoologických zahrad, v chovech exotického ptactva, na různých ptačích farmách a u pernaté zvěře chované mysliveckými spolky.
- Prokázat hostitelskou specifitu a infektivitu různých druhů a genotypů kryptosporidií pomocí experimentálních infekcí.
- Popsat biologii získaných druhů a genotypů kryptosporidií, zejména průběh infekce, patogenitu a lokalizaci vývojového cyklu včetně popisu vývojových stádií.
- Popsat diverzitu kryptosporidií ptáků pomocí multilokusové genotypizace a fylogenetických analýz.

3. LITERÁRNÍ PŘEHLED

3.1 Třída ptáci

Ptáci patří mezi teplokrevné obratlovce a jsou jednou z nejrozmanitějších skupin moderních obratlovců. Ptáci se vyvinuli z teropodních dinosaurů během jury (asi před 165–150 miliony let) a jejich klasický malý, lehký, opeřený a okřídlený tělesný plán se skládal dohromady postupně během desítek milionů let evoluce. Raní ptáci se diverzifikovali v průběhu jury a křídy, stali se schopnými letci s rychlým růstem. Při masivním vymírání na konci křídy (pozdní druhohory) a palegónu (starší třetihory) vyhynula spolu se svými blízkými dinosauřími příbuznými i většina ptáků. Po tomto hromadném vymírání se moderní ptáci explozivně diverzifikovali do současných více než 10 000 druhů a obsadili různé ekologické niky napříč celým světem (Veselovský 2001, Brusatte et al. 2015).

Charakteristickým znakem většiny ptáků je adaptace stavby těla na let. Některé druhy ptáků schopnost létat ztratily. Jsou to především všichni běžci, jako je pštros (*Struthio*), emu (*Dromaius*), nandu (*Rhea*) a kivi (*Apteryx*). Tělo ptáků je pokryto peřím, které vyniká malou hmotností. Velmi složitý a fyzicky náročný způsob ptačího pohybu vyžaduje intenzivní tělesný metabolismus a velmi výkonný oběhový i dýchací systém (Kučerová-Pospísilová et Ditrich 1998). Ptačí kosti jsou duté, bez kostní dřeně, která se vyskytuje jen velmi omezeně v některých kostech a tím se výrazně snížila hmotnost. Díky tomu hmotnost kostry ptáků představuje jen kolem 4 % hmotnosti těla, u savců je to 15–30 %. S tímto souvisí i rozdělení tvorby buněk imunitního systému. Zatímco u savců se tvoří a zrají B lymfocyty právě v kostní dřeni, u ptáků se přesunula tvorba do Fabriciovy burzy. Právě díky nálezu B lymfocytů v burze získaly tyto lymfocyty svou zkratku (Glick et al. 1956, Tizard 1979, Sun et al. 2012, Jílek 2014, Staley et Bonneaud 2015).

3.1.1 Anatomie trávicího traktu ptáků

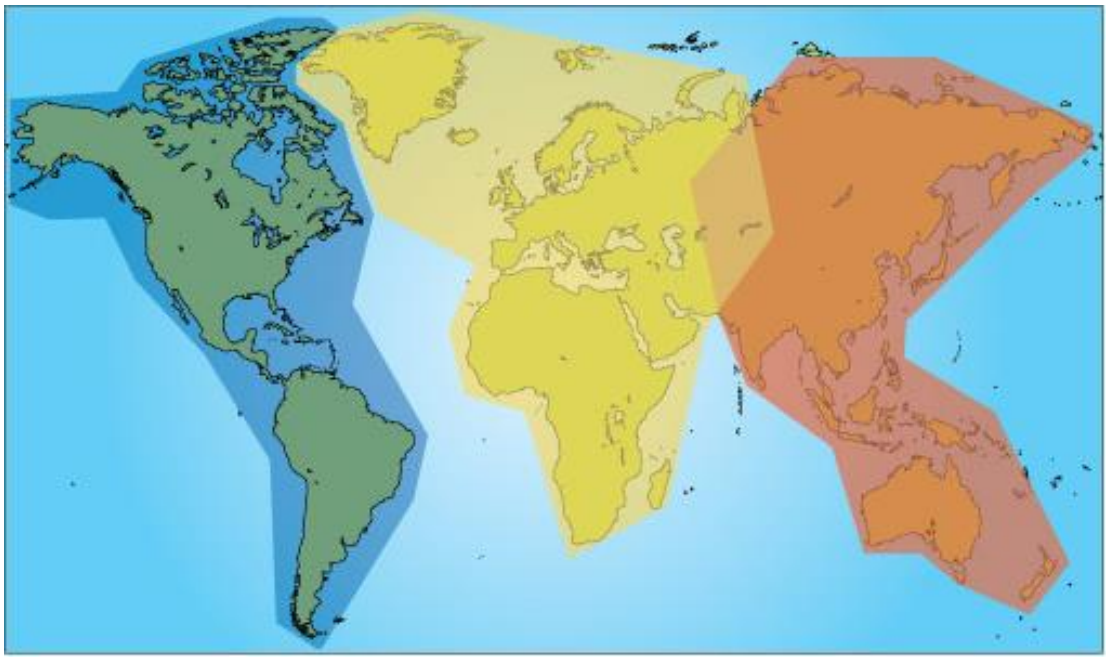
Trávicí soustava ptáků se podobá trávicí soustavě savců, ale existují mezi nimi některé zásadní rozdíly. Ptáci nemají zuby a potravu zpracovávají mechanicky zobákem a ve svalnatém žaludku. Jícen (*oesophagus*) se rozděluje na úsek před a za voletem. Jeho průměr je větší než u savců, což ptákům umožňuje polykat i velké kusy potravy, které by savci rozkousali na malé kusy. Vole (*ingluvies*) je vychlípenina jícnu a má především funkci skladovací. Nejlépe je vole vytvořeno u

semenožravých ptáků a některých rybožravých ptáků. U některých ptáků zcela chybí, např. u běžců funkci volete přejímá žláznatý žaludek. U hrabavých (Galliformes) a sokolů (Falconiformes) tvoří vole jednoduchý vak v místě vstupu do hrudníku. U papoušků (Psittaciformes) je vole uloženo napříč. U kanárů (*Serinus*) a vrubozobých ptáků (Anseriformes) se krční část jícnu pouze vřetenovitě rozšiřuje a tím vytváří náznak volete. U holubů (Columbidae) tvoří vole tři vakovité vychlípeniny, dvě postranní a jednu střední. Na rozdíl od polygastrických savců je žláznatý žaludek (*proventriculus*) uložen před svalnatým žaludkem. Žaludeční sekrece HCl, pepsinogenu a mucinu probíhá ve žláznatém žaludku, kde se potrava dlouho nezdržuje a pokračuje plynule do svalnatého žaludku (*ventriculus*), který je uzpůsoben pro mechanické zpracování potravy. U hmyzo- a masožravých ptáků se oddělují nestravitelné částí, které jsou vyvrhovány zpět ve formě vývržků. Někteří ptáci (běžci, hrabaví a někteří papoušci) polykají grit (drobné kamínky a písek), který ve svalnatém žaludku pomáhá rozmělňovat potravu. Tenké střevo, kde probíhá většina trávicích procesů a absorpce živin, má zřetelný dvanáctník (*duodenum*), v jehož kličce je uložena slinivka břišní. Hranice mezi lačníkem (*jejunum*) a kyčelníkem (*ileum*) však není patrná. Uprostřed délky tenkého střeva je patrný pozůstatek po žloutkovém váčku. Sliznice tenkého střeva je podobná jako u savců s tou výjimkou, že klky mají dobře vyvinuté krevní kapiláry, ale nemají centrální chylový kanálek. Slepá střeva jsou dvě a bývají různě velká. Jsou lokalizována na přechodu tenkého a tlustého střeva. Do slepých střev se nedostává všechna potrava. Nejvýznamnější funkcí slepých střev je mikrobiální zpracování celulózy. Moč, která se dostává do tračníku z kloaky, se může dostat až do slepých střev antiperistaltickými vlnami, které jsou největší zvláštností pohybů tračníku ptáků, a proto se slepá střeva neustále plní. Ve slepých střevech se kyselina močová stává zdrojem dusíku pro bakterie, které rozkládají celulózu. Další důležitou funkcí slepých střev je zpětná resorpce vody z moči. Trávicí soustava ptáků končí kloakou, která je společným vývodem pro trávicí, pohlavní a močovou soustavu. Fabriciova burza je dorzálně umístěný váček ve stěně proktodea, ve kterém dozrávají B-lymfocyty (Altman et al. 1997, Černý 2005, Reece 2011).

3.1.2 Migrace ptáků

Migrace je přesun z jednoho místa na druhé, konkrétně jde o přesun z hnízdiště na zimoviště a zpět (Berthold 2001). Migrace bezprostředně souvisí s fotoperiodicitou ptačích populací, vnitřními hodinami a se schopností ptáků se orientovat v prostředí. Díky vynikající pohyblivosti a jejich letovým schopnostem mohou ptáci každoročně překonávat i pozoruhodně dlouhé cesty z areálu hnízdění do zimovišť. Ptáky, kteří migrují, lze rozdělit na migranty na krátké nebo dlouhé vzdálenosti. Ptáci migrující na krátké vzdálenosti obvykle létají po souši v rámci kontinentů. Naopak ptáci migrující na velké vzdálenosti překračují hranice kontinentů a často i moře a oceány. Toto rozdělení je však jen základní, protože obě skupiny se vzájemně prolínají a závisí i na variabilitě migrační vzdálenosti. Rozlišujeme tři hlavní tahové systémy – paleakrticko-afrotropický, neoarkticko-neotropický a asijsko-australskoasijský (Newton 2006; Obrázek 1). Podle radarových záznamů každý rok odlétá více jak osm miliard ptáků ze severní polokoule na svá zimoviště v Africe a Jižní a Střední Americe (Boere et al. 2006).

Obrázek 1. Tři hlavní světové trasy migrace ptáků. Neoarkticko-neotropická trasa - modrá; Paleakrticko-afrotropická trasa - žlutá; Asijsko-australskoasijská trasa - červená (převzato z Migration: 2.1 Migration paths and flyways - OpenLearn - Open University - S295_1).



Schopnost ptáků létat a překonávat značné vzdálenosti usnadňuje přenos patogenů zahrnující velké množství původců virových (např. virus ptačí chřipky, newcastleská choroba, ptačí pneumoviry, kachní virus, riketsie, západonilská horečka nebo virus koňské encefalomyelitidy), bakteriálních (např. borelie, chlamydie, kampylobaktery, pasterely či salmonely) nebo parazitárních onemocnění (bičenky, hlístice, kryptosporidie) (Hoogstraal 1979, Jourdain et al. 2007, Dhama et al. 2009, Takekawa et al. 2010, Lawson et al. 2011, Prosser et al. 2011, Rumer et al. 2011, Chitimia-Dobler et al. 2019, Hubálek et al. 2020). Vyjma původců onemocnění, kteří jsou specifičtí pouze pro ptáky, představují ptáci významný zdroj patogenních organismů přenosných na člověka a jím chovaná hospodářská zvířata (Reed et al. 2003). Kromě toho se ptáci mohou stát dálkovými přenašeči organismů odolných vůči lékům a podílet se na vzniku nových endemických ohnisek nákaz podél migračních tras. Může tak docházet k objevení již známých infekcí v nových populacích, k rychlejšímu šíření nebo rozšiřovaní geografického výskytu (Reed et al. 2003).

Šířením jednotlivých druhů kryptosporidií prostřednictvím migrujících ptáků se zatím podrobně nikdo detailněji nezabýval. Největší druhová diverzita kryptosporidií byla zaznamenána u pěvců, papoušků a vrubozobých (Wang et al. 2021), nicméně v literatuře nelze nalézt mnoho indicií, které by mohly ukazovat na přímé šíření těchto parazitů ptačími hostiteli napříč geografickými oblastmi. Kromě toho, u celé řady genotypů (*Cryptosporidium* sp. avian IV, VI–IX, *Cryptosporidium* sp. canary genotyp I a II, *Cryptosporidium* sp. finch genotyp I, II a III, *Cryptosporidium* sp. Eurasian woodcock genotyp, *Cryptosporidium* sp. genotyp YS-2017) nejsou známy žádné informace o jejich rozšíření a hostitelské specifitě. Tyto genotypy byly popsány pouze u jednoho nebo několika málo jedinců v rámci několika publikací (Morgan et al. 2001, Ryan et al. 2003a, Ng et al. 2006, Gomes et al. 2012, Chelladurai et al. 2016, Helmy et al. 2017, Makino et al. 2018).

3.2 Kryptosporidie a kryptosporidióza ptáků

3.2.1 Historie a evoluce kryptosporidií

Odpověď na otázku „Jak jsou kryptosporidie staré“ a kdy se evolučně oddělily od svého nejbližšího příbuzného, není zcela jasná. Garcia et Hayman (2016) se pokusili zrekonstruovat vývoj rodu *Cryptosporidium* pomocí molekulárních hodin. Výsledky jejich studie ukázaly, že kryptosporidie se pravděpodobně oddělily od svého předka někdy před 590 miliony lety (877–345) a v období středních prvohor se rozdělily na dvě samostatné skupiny. K formování linie zahrnující druhy *C. muris, C. andersoni, C. serpentis, C. fragile* a *C. galli*, tedy druhy osidlující žaludek svých hostitelů, došlo pravděpodobně před 368 miliony let (560–218), zatímco k formování linie zahrnující druhy *C. baileyi, C. bovis, C. canis, C. cuniculus, C. erinacei, C. fayeri, C. felis, C. hominis, C. macropodum, C. meleagridis, C. molnari, C. parvum, C. ryanae, C. scrofarum, C. suis, C. tyzzeri, C. ubiquitum, C. varanii, C. viatorum, C. wrairi* a *C. xiaoi* (poznámka – druhy použité ve studii) infikující střeva svých hostitelů došlo později, přibližně před 265 miliony let (409–153). Tyto časy vzniku jednotlivých linií kryptosporidií se překrývají s intervalem odhadů věků uváděných pro původ obratlovců. Věk oddělení žaludeční a střevní linie je také v souladu s dobou, kdy se začínaly vyskytovat první druhy ryb (Kumar et Hedges 1998, Blair et Hedges 2005, Erwin et al. 2011, Hedges et al. 2015). Nicméně je nutné poznamenat, že druh *Cryptosporidium* "*struthionis*", podle kterého byla evoluce kryptosporidií mapována a který byl izolován ze pštrosa a jehož příbuzné kmeny byly nalezeny ve zkamenělinách ptáků Moa (Wood et al. 2013), v přílivových a odlivových usazeninách (Wilms et al. 2006) a odpadní vodě z lodí (Lohan et al. 2017) není platným druhem rodu *Cryptosporidium* a s největší pravděpodobností se nejedná o kryptosporidii, ale jiný kryptosporidiím příbuzný organismus.

První zmínka o kryptosporidiích pochází až z roku 1907, kdy Ernest Edward Tyzzer popsal vývojový cyklus parazitů, které nalezl ve sliznici žaludku laboratorních myší a popsal je jako nový druh, který pojmenoval *Cryptosporidium muris* (Tyzzer 1907, 1910). Tyzzer pravděpodobně nebyl první, kdo kryptosporidie dekoval. V letech 1894–95 popsal Clark výskyt parazitů ve sliznici žaludku myší nápadně podobným *C. muris*, nicméně je považoval za *Coccidium falciforme* (dnes *Eimeria falciformis*) parazitující běžně u myší ve střevě (Clark 1894-95). V roce

1912 popsal Tyzzer další druh kryptosporidií s vývojovým cyklem v tenkém střevě myší a pojmenoval ho *Cryptosporidium parvum* (Tyzzer 1912). První kryptosporidie u ptáků byly popsány v roce 1929 Tyzzerem ve střevě kuřat (Tyzzer 1929), nicméně až v roce 1955 popsal Slavin v pořadí třetí druh rodu *Cryptosporidium*. Tento druh, který detekoval u krůťat pojmenoval *Cryptosporidium meleagridis* (Slavin 1955). Do počátku 70. let 20. století nebyly kryptosporidie považovány za lékařsky či veterinárně významné a výzkum kryptosporidií nebyl v tomto období intenzivně prováděn (Wetzel 1938, Panciera et al. 1971, Meuten et al. 1974, Nime et al. 1976, Lasser et al. 1979). V sedmdesátých letech 20. století se kryptosporidie staly středem pozornosti mnoha studií, když byly identifikovány jako příčina vyčerpávajících a život ohrožujících vodnatých průjmů telat a jehňat, které se nedařilo vyléčit žádnými chemoterapeutiky a často končily úhynem (Panciera et al. 1971). Ve stejném období byla u ovcí, ještěrů, sluk a hus popsána řada druhů kryptosporidií, které jsou dnes považovány za neplatné (např. *C. agni*, *C. ameivae*, *C. baikalika*, *C. ctenosauris*, *C. anserinum*) nebo byly znovu nově popsány (např. *C. bovis*, *C. tyzzeri*, *C. cuniculus*) až koncem 20. a začátkem 21. století s nástupem molekulárních metod (Carreno et al. 1999, Sulaiman et al. 1999). Zájem o kryptosporidie vzrostl až v roce 1993, a to v souvislosti s masivní kontaminací vody oocystami kryptosporidií (*C. hominis*), kdy se nakazilo více než 400 000 osob v Milwaukee ve Wisconsinu (Mac Kenzie et al. 1994). V současné době jsou kryptosporidie považovány za významné lidské a zvířecí patogeny (Sréter et Varga 2000, Seva Ada et al. 2011, Eibach et al. 2015). Pro přehlednost níže uvádím chronologický popis jednotlivých druhů a genotypů ptačích kryptosporidií. V přehledu nejsou uvedeny druhy a genotypy, které jsou prokazatelně hostitelsky specifické pro jiné obratlovce, přestože u ptáků byly příležitostně nalezeny (více v kapitole 3.2.7).

Chronologie popisu druhů a genotypů kryptosporidií ptáků

1929 První zmínka o kryptosporidiích u ptačích hostitelů

Tyzzer (1929) detekoval kryptosporidie velmi podobné druhu *C. parvum* v céku kuřat.

1947 *Cryptosporidium baikalika* nom. nud.

Matschoulsky (1947) popsal *Cryptosporidium baikalika* nom. nud. jako nový druh kryptosporidie u sluky (*Scolopax*). Tento druh není v současné době platný. Publikované nákresy naznačují, že se jednalo o oocysty gregarin.

1955 *Cryptosporidium meleagridis* sp. n.

Slavin (1955) detekoval kryptosporidie parazitující v tenkém střevě krůt (*Meleagris gallopavo* f. *domestica*), popsal morfologii oocyst a dalších vývojových stadií a pojmenoval tento druh jako *Cryptosporidium meleagridis*.

1972 *Cryptosporidium* sp. u jestřába lesního

Gottschalk (1972) popsal *Cryptosporidium* sp. u jestřába lesního (*Accipiter gentilis*). Pravděpodobně se jednalo o *Sarcocystis* sp.

1974 *Cryptosporidium anserinum* nom. nud.

Proctor et Kemp (1974) popsali nový druh *Cryptosporidium anserinum* nom. nud. nalezený v tlustém střevě hus (*Anser domesticus*). Morfologie vývojových stádií nebyla publikovaná a tento druh je dnes považován za neplatný.

1982 *Cryptosporidium* sp. v dýchacím a zažívacím traktu křepelky

Tham et al. (1982) popsali výskyt a patogenitu *Cryptosporidium* sp. v dýchacím a zažívacím traktu křepelky (*Coturnix*). Žádné bližší informace o vývojovém cyklu a morfologii vývojových stádii nebyly uveřejněny.

1986 *Cryptosporidium baileyi* sp. n.

Current et Reese (1986) detailně popsali průběh infekce a vývojový cyklus včetně morfologie jednotlivých vývojových stádií kryptosporidie detekované v tenkém a tlustém střevě, kloace a Fabriciově burze kura domácího (*Gallus gallus* f. *domestica*) a pojmenovali tento druh *Cryptosporidium baileyi*.

1990 Tkáňová specifita *C. baileyi* a *Cryptosporidium blagburni* nom. nud.

Lindsay et Blagburn (1990) popsali další tkáňovou specifitu *C. baileyi* u přirozeně infikovaných ptáků. Kromě dříve detekovaných míst infekce (Current et Reese 1986) byl tento druh nalezen ve spojivkách, nosohltanu, průdušnici, plicích a vzdušných vacích, ledvinách a močovodu. Ve stejném roce popsali Blagburn et al.

(1990) nový druh *C. blagburni* nom. nud. z proventrikulu amadiny páskované (*Amadina fasciata*). Nicméně tento druh nebyl publikován s odpovídajícím popisem, a proto není považován za platný druh.

1993/94 *Cryptosporidium* sp. u pštrosů

Gajadhar (1993) a Bezuidenhout et al. (1993) nezávisle na sobě popsali výskyt *Cryptosporidium* sp. u pštrosů. Gajadhar (1994) navázal na svou předchozí práci a popsal morfologii oocyst nalezených u pštrosů. Výsledky jeho práce naznačovaly, že *Cryptosporidium* sp. popsané u pštrosů je odlišné od do té doby známých ptačích kryptosporidií.

1999 *Cryptosporidium galli* sp. n.

Pavlásek (1999) nalezl morfologicky identickou kryptosporidii s *C. blagburni* nom. nud. v proventrikulu drůbeže a pěvců a pojmenoval ji *Cryptosporidium galli*.

2001 *Cryptosporidium* sp. finch genotyp I–III a *Cryptosporidium* sp. duck genotyp I a II

Rok 2001 lze považovat za počátek molekulární éry v detekci a genotypizaci kryptosporidií ptáků. Morgan et al. (2001) detekovali pomocí molekulárních metod *Cryptosporidium* sp. finch genotyp I, II a III u amadiny Gouldové (*Erythrura gouldiae*). Prokázali, že tyto izoláty se molekulárně liší od platných druhů kryptosporidií. Ve stejném roce byl publikován nález *Cryptosporidium* sp. duck genotyp I a II u kachny černé (*Anas rubripes*) a husy kanadské (*Branta canadensis*) (Jellison et al. 2004, Zhou et al. 2004).

2003 *Cryptosporidium* sp. Eurasian woodcock genotyp

Ryan et al. (2003a) detekovali a molekulárně odlišili *Cryptosporidium* sp. Eurasian woodcock genotyp ze sluky lesní (*Scolopax rusticola*) od ostatních druhů a genotypů kryptosporidií. Současně byla provedena redeskripce popisu druhu *Cryptosporidium galli*.

2004 *Cryptosporidium* sp. goose genotyp I–IV

Zhou et al. (2004) na základě molekulární genotypizace popsali ve vzorcích trusu husy kanadské *Cryptosporidium* sp. goose genotyp I a II. V stejném roce byly ze stejného hostitele popsány další dva genotypy - *Cryptosporidium* sp. goose genotyp III a IV (Jellison et al. 2004).

2006 *Cryptosporidium* sp. avian genotyp I–IV

Ng et al. (2006) ve své studii publikovali nález *Cryptosporidium* sp. avian genotyp I u kanára divokého (*Serinus canaria*) a páva korunkatého (*Pavo cristatus*). Ve stejném roce byly pomocí molekulárních analýz popsány další pro ptáky specifické genotypy kryptosporidií. *Cryptosporidium* sp. avian genotyp II byl nalezen u papoušků v Austrálii a pštrosů v Brazílii, *Cryptosporidium* sp. avian genotyp III papoušků v Austrálii (Ng et al. 2006) a *Cryptosporidium* sp. avian genotyp IV u kruhoočka japonského (*Zosterops japonicus*) v České republice (Ng et al. 2006).

2011 *Cryptosporidium* sp. avian genotyp V

Qi et al. (2011) pomocí molekulární detekce identifikovali *Cryptosporidium* sp. avian genotyp V u korely chocholaté (*Nymphicus hollandicus*) v Číně.

2012 *Cryptosporidium* sp. canary genotyp I a II, *Cryptosporidium* sp. genotyp Jawa

Gomes et al. (2012) na základě genotypizace popsali tři nové genotypy kryptosporidií. U kanára divokého (*Serinus canaria*) popsali *Cryptosporidium* sp. canary genotyp I a II a u chůvičky japonské (*Lonchura striata domestica*) *Cryptosporidium* sp. genotyp Jawa.

2016 *Cryptosporidium* sp. duck genotyp IIb, goose genotyp Id a avian genotyp VI

V tomto roce byl publikován nález *Cryptosporidium* sp. duck genotyp IIb a *Cryptosporidium* sp. goose genotyp Id u vodních ptáků v severním Španělsku (Cano et al. 2016), a *Cryptosporidium* sp. avian genotyp VI u vlhovce červenokřídlého (*Agelaius phoeniceus*) v USA (Chelladurai et al. 2016) (**příloha I**).

2016 *Cryptosporidium avium* sp. n. (dříve *Cryptosporidium* sp. avian genotyp IV)

Holubová et al. (2016) popsali morfologické, molekulární a biologické vlastnosti *Cryptosporidium* sp. avian genotyp IV a navrhli tuto kryptosporidii popsat jako samostatný druh *Cryptosporidium avium* **(příloha II)**

2017 *Cryptosporidium* sp. avian genotyp VII–IX

Helmy et al. (2017) popsali další tři genotypy ptačích kryptosporidií u krocanů a kuřat v Německu a nazvali je *Cryptosporidium* sp. avian genotyp VII, VIII a IX.

2018 Makino et al. (2018) nalezli u puštíka hnědého (*Strix leptogrammica*) v Japonsku další molekulárně odlišný genotyp kryptosporidií, který nazvali *Cryptosporidium* sp. genotyp YS-2017.

2019 *Cryptosporidium proventriculi* sp. n. (dříve *Cryptosporidium* sp. avian genotyp III)

Holubová et al. (2019) popsali morfologické, biologické a molekulární vlastnosti *Cryptosporidium* sp. avian genotyp III a navrhli tuto kryptosporidii popsat jako samostatný druh *Cryptosporidium proventriculi* (**příloha V**).

2020 *Cryptosporidium ornithophilus* sp. n. (dříve *Cryptosporidium* sp. avian genotyp II)

Holubová et al. (2020) popsali morfologické, biologické a molekulární vlastnosti *Cryptosporidium* sp. avian genotyp II a navrhli tuto kryptosporidii popsat jako samostatný druh *Cryptosporidium ornithophilus* (**příloha VI**).

3.2.2 Historie a evoluce kryptosporidií

Kryptosporidie jsou řazeny do kmene Apicomplexa, v rámci něhož byly dlouhou dobu zařazeny mezi kokcidie (intracelulární, často hostitelsky specifičtí paraziti střev a dalších orgánů obratlovců prodělávající nepohlavní a pohlavní vývoj zakončený produkcí velmi odolných oocyst, které jsou z hostitele nejčastěji vylučovány trusem), zejména díky morfologické podobnosti vývojových stadií a vývojovému cyklu v gastrointestinálním traktu (Fayer et Xiao 1997). Nicméně taxonomické postavení kryptosporidií bylo vždy předmětem diskuzí. Již Tyzzer, který kryptosporidie popsal a ke kokcidiím je přiřadil, poznamenal, že se od kokcidií liší stavbou oocyst, způsobem přichycení na hostitelskou buňku a schopností autoinfekce (Tyzzer 1910). Také úplná nevnímavost na léčbu veškerými antikokcidiky naznačovala nesprávné zařazení těchto parazitů ke kokcidiím (Abrahamsen et al. 2004). Další důkaz o nejistém postavení kryptosporidií v rámci kokcidií přinesly výsledky antigenní příbuznosti zjištěné monoklonálními protilátkami (Bull et al. 1998). Zásadní zlom v taxonomickém zařazení těchto parazitických prvoků přinesly molekulární analýzy, které prokázaly bližší příbuznost kryptosporidií s gregarinami (Gregarinasina) než s kokcidiemi (Coccidiasina) (Carreno et al. 1999, Ryan et al. 2016). Tento fakt byl dále podpořen i podobnou

morfologickou stavbou organel sloužících k přichycení k hostitelské buňce (Valigurová et al. 2007) a nepřítomností plastidového genomu (Zhu et al. 2000). Na základě těchto skutečností byly kryptosporidie přesunuty z podtřídy Coccidia do nové podtřídy Cryptogregaria v rámci nové třídy Gregarinomorphea a nového řádu Cryptogregarida (Cavalier-Smith 2014).

Do současné doby bylo popsáno 49 platných druhů kryptosporidií a detekovány stovky genotypů, které byly od platných druhů odlišeny na základě hostitelské specificity a v posledních 20 letech mnohem častěji na základě molekulární odlišností. Většina genotypů byla nejčastěji popsána na základě odlišností v sekvenci genu kódujícího malou podjednotku rRNA (Kváč et al. 2014b). Morfologické rozdíly ve velikosti a tvaru oocyst jsou u většiny kryptosporidií natolik malé, že je nelze využít jako diferenciální znak pro odlišení druhu (Jex et al. 2008, Kváč et al. 2013a, Kváč et al. 2014a).

3.2.3 Vývojový cyklus kryptosporidií

Kryptosporidie jsou paraziti, kteří ukončují svůj vývojový cyklus v jednom hostiteli (monoxenní cyklus). Většina kryptosporidií je, až na pár výjimek, hostitelsky specifická (viz kapitola 3.2.7). Predilekčním místem infekce jsou epiteliální buňky zažívacího traktu, nicméně u některých druhů parazitujících u ptáků byl popsán výskyt i v dýchacím a urogenitálním traktu (Current et Blagburn 1990). Jen omezené množství druhů a genotypů kryptosporidií infikuje více než jedno specifické místo (kapitola 3.2.4). Přestože dnes známe velké množství druhů a genotypů kryptosporidií, vývojový cyklus byl popsán pouze u pěti druhů, a to u tří druhů savčích kryptosporidií, *C. parvum* (Tyzzer 1912), *C. muris* (Tyzzer 1907, 1910) a *C. proliferans* (Melicherová et al. 2013) a dvou ptačích druhů, *C. baileyi* (Current et al. 1986) a *C. ornithophilus* jehož vývojový cyklus byl popsán v rámci této práce (Holubová et al. 2020).

Vývojový cyklus lze rozdělit do čtyř hlavních fází: excystace, merogonie (nepohlavní rozmnožování), gametogonie (pohlavní rozmnožování) a sporogonie (Fayer et Xiao 1997, Thompson et al. 2005). Oocysty kryptosporidií vylučované z hostitele trusem/stolicí, sputem nebo močí jsou na rozdíl od kokcidií plně infekční ihned po vyloučení z těla hostitele. Po pozření oocysty hostitelem dochází k excystaci, kdy se uvolní čtyři infekční sporozoiti, kteří infikují epiteliální buňky (Fayer 2007). Proces excystace je zásadní pro rozvoj infekce. Každý druh/genotyp

kryptosporidie excystuje v přesně daném místě zažívacího traktu. Například *C. andersoni* infikuje výlučně slez skotu, *C. parvum* parazituje v tenkém střevě, zejména v duodenu a jejunu, *Cryptosporidium* sp. chipmunk genotyp I osidluje výhradně slepé střevo a vývoj druhu *C. occultus* je lokalizován pouze v tlustém střevě (Lindsay et al. 2000, Widmer et al. 2007, Stark et al. 2009, Matsubayashi et al. 2011, Modrý et al. 2012, Kváč et al. 2016, Kváč et al. 2018). Uvolnění sporozoiti se apikálním koncem přichycují k povrchu epiteliálních buněk, morfologicky se mění na trofozoity a prodlužováním a spojováním mikrovilární vrstvy epitelu dochází k tvorbě parazitoforního vaku, ve kterém je trofozoit uzavřen (Elliott et al. 2001, Valigurová et al. 2007, Melicherová et al. 2013). Z trofozoita vzniká meront, jehož jádro se v průběhu merogonie dělí a vznikají meronti, jejichž počet je dán typem merogonie. Při první merogonii vzniká meront I. typu jehož jádro se dělí na 6–8 jader/merozoitů. Někteří merozoiti I. typu dávají vzniknout opět merontům I. typu a ostatní se přeměňují na meronty II. typu, kteří tvoří pouze 4 merozoity (Aydin et Ozkul 1996, Hijjawi 2010, Melicherová et al. 2013). Vznikem merozoitů II. typu je ukončena fáze merogonie (Current et al. 1986, Hijjawi 2010). U druhu *C. baileyi*, byl popsán ještě třetí typ merontů, který dává vzniknout 8 merozoitům III. typu (Current et al. 1986). Meronti II., respektive III. typu dávají vzniknout vícejaderným mikrogamontům a jednojaderným makrogamontům, čímž začíná fáze gametogonie. Z mikrogamontů se uvolňují pohyblivé mikrogamety (16), které migrují a oplodňují makrogamety vzniklé přeměnou makrogamontů, čímž vzniká zygota, která prochází sporogonií, během které vznikají oocysty dvojího typu (Sunnotel et al. 2006, Hijjawi et al. 2010). Většina oocyst je tzv. silnostěnných (80 %), které jsou vylučovány z těla hostitele a slouží k infekci dalších vnímavých jedinců. Menší část (20 %) oocyst je tenkostěnných, které ve většině případů tělo hostitele neopouští, excystují v hostiteli a způsobují autoinfekci (Current et Reese 1986).

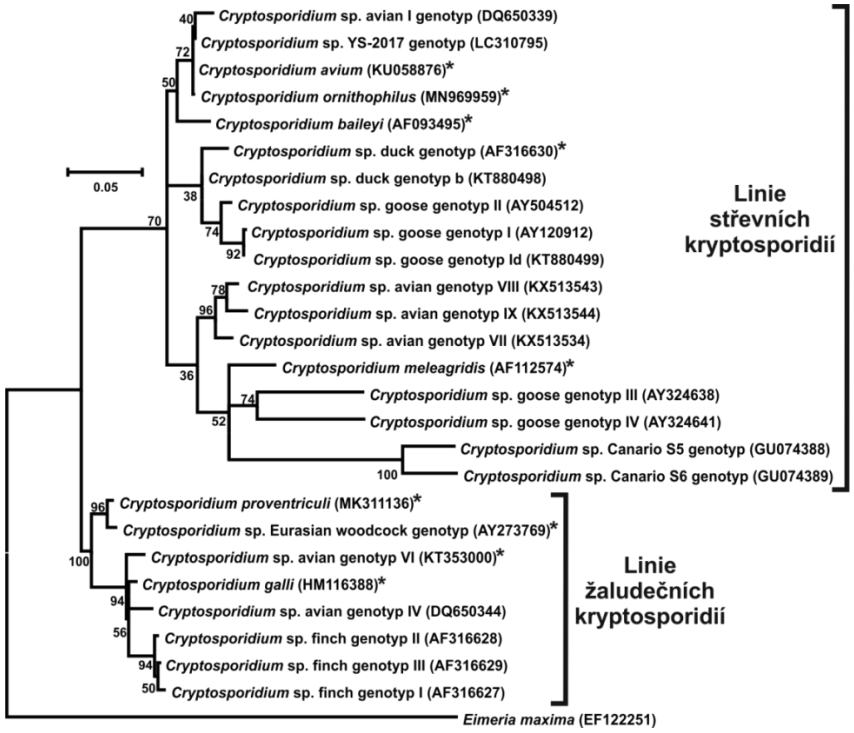
3.2.4 Orgánová a tkáňová specifita ptačích kryptosporidií

Jak již bylo výše naznačeno, jednotlivé druhy/genotypy kryptosporidií se vyznačují tkáňovou/orgánovou specifitou. Vývoj všech dosud popsaných kryptosporidií je vždy lokalizován do některé části zažívací soustavy s možností infekce dalších orgánových soustav. Podle místa infekce lze kryptosporidie rozdělit do dvou monofyletických odlišných linií (Xiao et al. 2004a; Tabulka 1). První, fylogeneticky starší linií jsou kryptosporidie infikující epiteliální buňky žláznatého a svalnatého

žaludku svých hostitelů (Kváč et al. 2013b) a druhou linií jsou střevní kryptosporidie (Xiao et al. 2002). V rámci střevní linie se pak jednotlivé druhy dělí na kryptosporidie infikující tenké, slepé a tlusté střevo. Většina kryptosporidií, u kterých je lokalizace známa, se vyvíjí v tenkém střevě, nejčastěji v duodenu a jejunu (Čondlová et al. 2018, Horčičková et al. 2019, Ježková et al. 2021b). Jen u malé části druhů/genotypů byl popsán vývoj ve slepém nebo tlustém střevě (Čondlová et al. 2018, Kváč et al. 2018, Horčičková et al. 2019). Pro druhy *C. baileyi* a *C. avium,* které jsou hostitelsky adaptovány na ptáky, je charakteristická multiorgánová lokalizace. Druh *C. baileyi*, který je primárně lokalizován v tenkém střevě, je často spojován s infekcí kloaky, spojivek, plic, ledvin a Fabriciovy burzy (Current et al. 1986). Také *C. avium,* jehož predilekčním místem infekce je ileum a cékum, byl nalezen v ledvinách a močovodech (Curtiss et al. 2015, Holubová et al. 2016). Orgánová a tkáňová afinita není známa pro všechny dosud popsané druhy a genotypy kryptosporidií. Lokalizace v hostiteli je často odvozována na základě příslušnosti k fylogenetické linii (Obrázek 2).

Primární (predilekční) a sekundární místa infekce jednotlivých druhů a genotypů ptačích kryptosporidií se mohou překrývat, a proto je nelze využít jako spolehlivý diferenciální znak (Tabulka 1).

Obrázek 2. Příslušnost jednotlivých druhů a genotypů kryptosporidií ptáků ke střevní a žaludeční linii. Druhy a genotypy, u kterých byla lokalizace infekce potvrzena, jsou označeny hvězdičkou (*). GenBank registrační číslo sekvence je uvedeno v závorce.



Tabulka 1. Primární (predilekční) a sekundární místa infekce jednotlivých druhů a genotypů ptačích kryptosporidií, u kterých byla lokalizace ověřena.

***Cryptosporidium* spp.**	**Lokalizace**		**Reference**
	primární	**sekundární**	
C. avium	ileum a cékum	močovod, ledviny	Holubová et al. (2016)
C. baileyi	tenké střevo, cékum, tlusté střevo, kloaka, Fabriciova burza	oko, průdušnice, nosohltan, plíce, vzdušné vaky	Current et al. (1986)
C. galli	provetrikulus	-	Mathis et al. (1999), Ryan et al. (2003b)
C. meleagridis	ileum, cékum, tlusté střevo, kloaka, Fabriciova burza	-	Slavin (1955)
C. ornithophilus	cékum, tlusté střevo, kloaka, Fabriciova burza	-	Holubová et al. (2020)
C. proventriculi	provetrikulus a ventrikulus	-	Holubová et al. (2019)
***Cryptosporidium* sp. avian genotyp VI**	provetrikulus	-	Chelladurai et al. (2016)
***Cryptosporidium* sp. duck genotyp**	tenké střevo	-	Morgan et al. (2001)
***Cryptosporidium* sp. Euroasian woodcock genotyp**	provetrikulus	-	Ryan et al. (2003a)

3.2.5 Přenos infekce

Oocysty kryptosporidií jsou velmi odolné vůči nepříznivým podmínkám vnějšího prostředí (Fayer 2007). Zejména v chladném a vlhkém prostředí jsou schopné přežít mnoho měsíců (Fayer 2004). Navíc běžné dezinfekční prostředky, zejména na bázi chloru nejsou účinné (Fayer 2004, Baldursson et Karanis 2011, Burnet et al. 2014). V laboratorních podmínkách byla testována celá řada faktorů, jako jsou nízké a vysoké teploty, ultrafialové záření, ozonizace a dezinfekce atd., ovlivňující životaschopnost kryptosporidií (Anderson 1985, Robertson et al. 1992, Rhee et al. 1996, Slifko et al. 1997, Fayer et al. 1998, Jenkins et al. 2000, Walker et al. 2001, Jenkins et al. 2003, Surl et al. 2003, Fayer 2004, Kváč et al. 2007a). Stěžejním faktorem ovlivňujícím životaschopnost oocyst v prostředí je teplota. Optimální teplota je 5–15 °C (Fayer et al. 1998), při poklesu nebo vzestupu teploty dochází v důsledku rychlejšího metabolismu ke zkracování doby přežívání v prostředí (Jenkins et al. 2003, King et al. 2005). Expozice oocyst teplotám nad 55 °C způsobuje rychlou devitalizaci (Fayer 1994, Harp et al. 1996, Fujino et al. 2002, Neumayerová et Koudela 2008).

Přestože některé druhy kryptosporidií infikují i jiné orgány a orgánové soustavy než trávicí trakt (kapitola 3.2.4), jsou oocysty primárně vylučovány z hostitele trusem (Xiao 2010). Hostitelé mohou být infikováni buď přímo, tedy kontaktem s infikovaným jedincem (Ziegler et al. 2007, Feng 2010), nebo nepřímo, kontaminovanou vodou či potravou (Nyachuba 2010, Budu-Amoako et al. 2011). Sponseller et al. (2014) popsal přenos prostřednictvím kapénkové infekce - inhalací oocyst kryptosporidií. Lindsay et Blagburn (1986) v roce 1986 popsali experimentální infekci kuřat *Cryptosporidium* sp. přes kloaku. Jakým způsobem jsou ptáci nejčastěji infikováni, není známo, ale předpokládá se, že stejně jako u lidí a dalších obratlovců převládá infekce kontaminovanou potravou a vodou (Baldursson et Karanis 2011).

Vzhledem k tomu, že je voda považována za nejčastější zdroj oocyst kryptosporidií, dalo by se předpokládat, že většina druhů/genotypů kryptosporidií parazitujících u zvířat vázaných na vodní prostředí, bude detekována v povrchových vodách. Zajímavé je, že většina kryptosporidií ptáků, zvláště těch, které jsou specifické pro vodní ptáky, nebyla v povrchových vodách detekována (Tabulka 2).

Tabulka 2. Seznam druhů a genotypů kryptosporidií detekovaných v povrchových (PV) a odpadních vodách (OV).

***Cryptosporidium* spp.**	**Typ vod**	**Stát (reference)**
C. andersoni	PV	**AUS** (Zahedi et al. 2020) ; **BEL** (Ehsan et al. 2015); **CAN** (Ruecker et al. 2005, Wilkes et al. 2013); **GBR** (Mcevoy et al. 2005, Robinson et al. 2011); **GRC** (Ligda et al. 2020); **CHN** (Ma et al. 2019**)**; **IRN** (Mahmoudi et al. 2015); **JPN** (Masago et al. 2006); **PRT** (Lobo et al. 2009); **ESP** (Kishida et al. 2012**)**
	OV	**CHN** (Liu et al. 2011, Ma et al. 2019); **USA** (Zhou et al. 2003)
C. baileyi	PV	**GBR** (Ruecker et al. 2005)
C. bovis	PV	**AUS** (Swaffer et al. 2014, Zahedi et al. 2020)
C. canis	PV	**AUS** (Bryan et al. 2009, Swaffer et al. 2014); **IRN** (Mahmoudi et al. 2015)
C. cuniculus	PV	**AUS** (Bryan et al. 2009, Swaffer et al. 2014, Zahedi et al. 2020)
C. fayeri	PV	**AUS** (Bryan et al. 2009, Swaffer et al. 2014)
C. fragile	PV	**NGA** (Falohun et al. 2021)
C. hominis	PV	**AUS** (Bryan et al. 2009, Loganthan et al. 2012, Zahedi et al. 2020); **BEL** (Ehsan et al. 2015); **BRA** (Araujo et al. 2011); **CHN** (Ma et al. 2019); **IRN** (Mahmoudi et al. 2015); **JPN** (Masago et al. 2006); **PRT** (Lobo et al. 2009); **USA** (Dreelin et al. 2014, Stokdyk et al. 2019)
	OV	**USA** (Zhou et al. 2003)
C. macropodum	PV	**AUS** (Bryan et al. 2009, Zahedi et al. 2020)
C. meleagridis	PV	**BRA** (Araujo et al. 2011)
C. muris	PV	**AUS** (Swaffer et al. 2014); **ESP** (Kishida et al. 2012); **IRN** (Mahmoudi et al. 2015); **NGA** (Falohun et al. 2021); **PRT** (Lobo et al. 2009)
	OV	**GRC** (Spanakos et al. 2015); **USA** (Zhou et al. 2003)
C. parvum	PV	**AUS** (Ryan et al. 2005, Bryan et al. 2009, Loganthan et al. 2012, Swaffer et al. 2014, Zahedi et al. 2020); **BEL** (Ehsan et al. 2015); **CAN** (Budu-Amoako et al. 2011); **GBR** (Mcevoy et al. 2005, Robinson et al. 2011); **HTI** (Damiani et al. 2013); **CHN** (Ma et al. 2019); **IRN** (Mahmoudi et al. 2015); **JPN** (Masago et al. 2006); **NGA** (Falohun et al. 2021); **PRK** (Bahk et al. 2018); **PRT** (Lobo et al. 2009); **USA** (Dreelin et al. 2014, Stokdyk et al. 2019)
	OV	**DEU** (Ajonina et al. 2012); **GRC** (Spanakos et al. 2015); **MYS**, **PHL**, **THA** (Kumar et al. 2016); **ROU** (Imre et al. 2017); **TUR** (Aslan et al. 2012); **USA** (Zhou et al. 2003)
C. ryanae	PV	**AUS** (Swaffer et al. 2014); **THA** (Chuah et al. 2016)
C. scrofarum	PV	**AUS** (Ryan et al. 2005)
C. suis	PV	**AUS** (Ryan et al. 2005); **BEL** (Ehsan et al. 2015)
C. tyzzeri	PV	**AUS** (Swaffer et al. 2014)
	OV	**USA** (Zhou et al. 2003)
C. ubiquitum	PV	**AUS** (Ryan et al. 2005, Bryan et al. 2009, Zahedi et al. 2020)
	OV	**CHN** (Liu et al. 2011); **USA** (Zhou et al. 2003)
C. xiaoi	PV	**AUS** (Ryan et al. 2005, Zahedi et al. 2020)
Cryptosporidium sp. horse genotyp	PV	**BEL** (Ehsan et al. 2015)
Cryptosporidium sp. muskrat genotyp I a II	PV	**CAN** (Wilkes et al. 2013)
Cryptosporidium sp. skunk genotyp	PV	**CAN** (Ruecker et al. 2005)

AUS: Austrálie; **BEL:** Belgie; **BRA:** Brazílie; **CAN:** Kanada; **DEU:** Německo; **CHN:** Čína; **ESP:** Španělsko; **GBR:** Spojené království Velká Británie a Severního Irska; **GRC:** Řecko; **HTI:** Haiti; **IRN:** Írán; **JPN:** Japonsko; **MYS:** Malajsie; **NGA:** Nigérie; **PHL:** Filipíny; **PRK:** Korejská lidově demokratická republika; **PRT:** Portugalsko; **ROU:** Rumunsko; **THA:** Thajsko; **TUR:** Turecko; **USA:** Spojené státy americké

3.2.6 Terapie a prevence

Dodnes neexistuje žádná účinná léčba nejen ptačí, ale obecně žádné kryptosporidiózy (Lindsay et Blagburn 1990, Fayer et Xiao 1997). V chovech ptáků se běžně používají na kryptosporidiózu antikokcidika samostatně nebo v kombinaci s Duokvinem (dihydroquinoline antioxidant). Experimentální studie ukázaly, že podávání těchto léčiv onemocnění způsobené *C. baileyi* neeliminuje, ani neslouží jako preventivní opatření (Lindsay et Blagburn 1990, Sréter et al. 1995). Další preparáty, oxytetracykliny, chlortetracykliny, aprolium a derivát pyrimidinu, neměly žádný efekt na respiratorní kryptosporidiózu u krocanů a pávů (Mason et Hartley 1980, Glisson et al. 1984), stejně tak preparát neomycin furazolidon nebyl účinný na střevní kryptosporidiózu u křepele virginského (*Colinus virginianus*) (Hoerr et al. 1986). U kuřat byla studována účinnost léčby kryptosporidiózy způsobené *C. baileyi*, zvláště na redukci počtu oocyst pomocí humánních léčiv rekombinantního interleukinu-1 beta (hrIL-1 beta) a prostaglandinu, inhibitoru indomethacinu. Parenterální aplikace tohoto přípravku snížila vylučování oocyst o 6 %, ale onemocnění mělo stejný průběh u skupiny kuřat bez aplikace léčiv a kuřat léčených. Nicméně pokud byl do krmiva přidáván přípravek Indomethacin došlo ke snížení počtu vylučovaných oocyst o 13,7 % a zkrácení doby infekce v porovnání s kontrolní skupinou (Hornok et al. 1999). Novější studie týkající se antikryptosporidiální profylaktické účinnosti enrofloxacinu a paramomycinu ukázala 52%, respektive 62% účinnost (Sréter et al. 2002). Všeobecně se při ptačí kryptosporidióze spoléhá na prevenci a zamezení zanesení infekčních oocyst z vnějšího prostředí.

3.2.7 Hostitelská specifita kryptosporidií

Hostitelská specifita vyjadřuje schopnost parazita infikovat a dokončit svůj vývojový cyklus v jednom či více hostitelích. K zjištění hostitelského spektra se využívají experimenty, kdy se infekčními oocystami kryptosporidií infikují odlišné druhy zvířat, než ze kterých byla infekční dávka získána. Jestliže hostitel po uplynutí doby nezbytné k dokončení vývojového cyklu začne vylučovat oocysty, které jsou geneticky shodné s oocystami z infekční dávky, lze považovat daného hostitele za vnímavého pro daný druh či genotyp kryptosporidií.

V rámci rodu *Cryptosporidium* můžeme na základě hostitelského spektra rozdělit druhy do dvou skupin. Do první skupiny, která je početně málo zastoupená, řadíme druhy, které jsou přirozeně infekční pro široké spektrum druhů hostitelů napříč

různými třídami a řády živočichů. Mezi zástupce této skupiny patří *C. parvum* a *C. ubiquitum*, *C. baileyi* a *C. meleagridis* (Tyzzer 1912, Slavin 1955, Li et al. 2014). Zástupci druhé skupiny jsou hostitelsky úzce specializovaní, a můžeme je rozdělit na dvě podskupiny. Do první podskupiny řadíme druhy, u kterých je znám pouze jediný hostitel nebo hostitelé patřící do jednoho rodu. Příkladem jsou *C. suis*, *C. wrairi* nebo *C. xiaoi* (Tyzzer 1910, Vetterling et al. 1971, Current et al. 1986, Fayer et Santín 2009). Do druhé podskupiny řadíme druhy, které infikují širší spektrem hostitelů v rámci fylogeneticky příbuzných rodů. Příkladem takovýchto kryptosporidií je *C. muris* parazitující u řady hlodavců nebo *C. andersoni* vyskytující se u zástupců turovitých (Bovidae) a velbloudovitých (Camelidae) (Koudela et al. 1998, Ryan et al. 2003a, Kváč et al. 2007b, Kváč et al. 2008).

Hostitelská specifita ptačích kryptosporidií je blíže popsána u jednotlivých druhů a genotypů v kapitole 3.2.8. Kromě hostitelsky specifických kryptosporidií byly u ptáků nalezeny i druhy, které jsou typické pro savce (Tabulka 3). Tyto případy můžeme vysvětlit náhodným pozřením oocyst a u ptáků byla zachycena pouze pasáž (Fayer et al. 2001, Xiao et al. 2007).

Tabulka 3. Savčí druhy kryptosporidií detekované u ptáků.

Druh	Typický hostitel	Ptačí hostitel (*vědecké jméno*)	Místo nálezu	Reference
C. andersoni	skot	koroptev korunkatá (*Rollulus rouloul*)	Evropa	Ng et al. (2006)
		labuť zpěvná (*Cygnus cygnus*)	Skotsko	Wang et al. (2019)
C. canis	psi	alexandr malý (*Psittacula krameri*)	Jižní Amerika	Ferrari et al. (2018)
C. hominis	člověk	vrubozobí berneška velká (*Branta canadensis*)	Severní Amerika	Santín et al. (2004)
			USA	Zhou et al. (2004)
C. muris	hlodavci	lelkoun soví (*Podargus strigoides*)	Asie	Ng et al. (2006), Qi et al. (2014)
		pštros dvouprstý (*Struthio camelus*)	Evropa	Qi et al. (2014)

Tabulka 3. Savčí druhy kryptosporidií detekované u ptáků (pokračování).

Druh	Typický hostitel	Ptačí hostitel (*vědecké jméno*)	Místo nálezu	Reference
C. parvum	skot	alexandr malý (*Psittacula krameri*)	Brazílie	Jamshidi et al. (2012)
		papoušíček (*Forpus* sp.)		
		dytík úhorní (*Burhinus oedicnemus*)	Dubaj	Zylan et al. (2008)
		korela chocholatá (*Nymphicus hollandicus*)	Brazílie	Nakamura et al. (2009)
		krahujec obecný (*Accipiter nisus*)	Španělsko	Diaz et al. (2015)
		káně lesní (*Buteo buteo*)		
		luňák hnědý (*Milvus migrans*)		
		včelojed lesní (*Pernis apivorus*),		
		straka obecná (*Pica pica*)		
		holub skalní (*Columba livia*)	Brazílie	Graczyk et al. (1998), Zhou et al. (2004), Zylan et al. (2008), Mcevoy et Giddings (2009), Nakamura et al. (2009), Gomes et al. (2012), Helmy et al. (2017), Oliveira et al. (2017a)
		kur domácí (*Gallus gallus* f. *domestica*)	Irán	Shahbazi et al. (2020)
		berneška velká (*Branta canadensis*)	USA	Graczyk et al. (1998)
		kur domácí (*Gallus gallus* f. *domestica*)	Německo	Helmy et al. (2017)
		krůta domácí (*Meleagris gallopavo* f. *domestica*)		
		chůvička japonská (Lonchura striata domestica)	Brazílie	Gomes et al. (2012)
		berneška velká (*Branta canadensis*)	USA	Zhou et al. (2004)
		krůta domácí (*Meleagris gallopavo* f. *domestica*)	USA	Mcevoy et Giddings (2009)

3.2.8 Druhy a genotypy kryptosporidií infikující ptáky

- ***Cryptosporidium meleagridis* Slavin, 1955**

Hostitelská specifita: Tato kryptosporidie není specifická pouze pro ptáky, ale běžně se vyskytuje i u savců včetně člověka (Wang et al. 2014). U ptáků byla infekce zaznamenána u ptáků z řádu papoušků, pěvců, hrabavých, měkkozobých a vrubozobých (Morgan et al. 2000c, Morgan et al. 2001, Ryan et al. 2003a, Huber et al. 2007, Wang et al. 2012, Baroudi et al. 2013, Wang et al. 2014, Li et al. 2015a, Máca et Pavlásek 2016). Díky experimentům bylo potvrzeno, že *C. meleagridis* je schopné infikovat velký okruh zvířat. Oocysty byly schopny infikovat brojlerová kuřata, kachny, krůty, telata, prasata, králíky, krysy a myši (O'donoghue 1985, Akiyoshi et al. 2003, Darabus et Olariu 2003, Huang et al. 2003).

Lokalizace a patogenita: O detailech průběhu infekce a lokalizaci vývojových stádií se ví překvapivě málo. Experimentálními infekcemi krůt oocystami *C. meleagridis* byla prokázána lokalizace vývojových stadií parazita v ileu, céku, kolonu a Fabriciově burze (Bermudez et al. 1988). Onemocnění je spojováno s průjmy a hubnutím (Slavin 1955, Gharagozlou et al. 2006). Tacconi et al. (2001) sledovali průběh infekce u 30–denního krocana a prokázali největší množství vývojových stádií *C. meleagridis* na epiteliálních buňkách střeva a Fabriciově burze. U infikovaných kuřat má infekce za následek nepatrné zkrácení střevních klků a nepravidelný povrch výstelkové vrstvy (Akiyoshi et al. 2003).

Fylogenetická analýza: Ještě na konci 20. století byly izoláty *C. meleagridis* získané z člověka považovány za genotyp druhu *C. parvum* (Champliaud et al. 1998, Sréter et Varga 2000). Na základě molekulárních analýz genů kódujících SSU, HSP70, COWP, aktin a gp60 u vzorků pocházejících z různých částí světa bylo prokázáno, že se jedná o samostatný druh (Xiao et al. 1999, Morgan et al. 2000b, Sréter et al. 2000b, Sulaiman et al. 2000, Xiao et al. 2000, Morgan et al. 2001, Sulaiman et al. 2002, Xiao et al. 2002, Leoni et al. 2006).

Morfologie a morfometrie oocyst: Oocysty jsou oválného tvaru. Velikost oocyst je 4,5–6,0 μm (průměr 5,2 μm) × 4,2–5,3 μm (průměr 5,3 μm) s poměrem délky a šířky 1,00–1,33 μm (Lindsay et al. 1989).

Věková specifita: Studií, které by se zabývaly věkovou variabilitou je velmi málo. Tůmová et al. (2002) sledovali infekci u brojlerových kuřat infikovaných v různém věku (7, 14 a 21 dní). Bez ohledu na věk, všechna zvířata trpěla typickými

příznaky a infekce neměla později vliv na úbytek hmotnosti ani úmrtnost sledovaných skupin ptáků.

Zoonotický potenciál: Výsledky mnoha studií potvrdily zoonotický potenciál *C. meleagridis*, zejména u lidí, žijících v blízkém kontaktu s chovanými ptáky (Silverlas et al. 2012, Widmer et al. 2015). *Cryptosporidium meleagridis* je třetí nejčastěji detekovanou kryptosporidií u lidí (Mclauchlin et al. 2000, Morgan et al. 2000a, Glaberman et al. 2001, Guyot et al. 2001, Pedraza-Diaz et al. 2001, Yagita et al. 2001, Enemark et al. 2002, Gatei et al. 2002, Tiangtip et Jongwutiwes 2002, Cama et al. 2003, Gatei et al. 2003, Leoni et al. 2003, Matos et al. 2004, Xiao et al. 2004a, Coupe et al. 2005, Gatei et al. 2006a, Gatei et al. 2006b, Muthusamy et al. 2006, Wang et al. 2014). *Cryptosporidium meleagridis* se nevyskytuje pouze u imunodeficitních pacientů, ale i u imunokompetentních jedinců (Leoni et al. 2006, Chalmers et al. 2009, Elwin et al. 2011, Wang et al. 2014). V roce 2019 bylo *C. meleagridis* prokázáno v adenokarcinomu tlustého střeva v Polsku u imunokompetentního pacienta, což prokázalo první důkaz vývoje *C. meleagridis* v rakovinotvorné tkáni (Kopacz et al. 2019), kdy doposud byl dříve prokázán vyšší výskyt *C. parvum* a *C. hominis* u pacientů s rakovinou tlustého střeva (Berahmat et al. 2017).

- ***Cryptosporidium baileyi* Current, 1986**

Hostitelská specifita: Tato ptačí kryptosporidie byla nalezena u ptáků řádu dlouhokřídlých (Charadriiformes), hrabavých (Galliformes), krátkokřídlých (Gruiformes), papoušků (Psittaciformes), pěvců (Passeriformes), pštrosů (Struthioniformes), šplhavců (Piciformes), veslonohých (Pelecaniformes) a vrubozobých (Anseriformes) (Lindsay et Blagburn 1990, Pavlásek 1993, Ryan et al. 2003a, Abe et Iseki 2004, Jellison et al. 2004, Kimura et al. 2004, Chvala et al. 2006).

Lokalizace a patogenita: Dle lokalizace se tento druh řadí mezi střevní kryptosporidie a jedná se o nejběžnější kryptosporidii u ptáků (Fayer 2007). Přirozená infekce u ptáků byla lokalizována nejen ve střevech, ale také ve spojivkách, v nosohltanu, průdušnici, průduškách, vzdušných vacích, Fabriciově burze, ledvinách a močovém traktu (Lindsay et al. 1987, Lindsay et Blagburn 1990, Abbassi et al. 1999). Mnoho experimentálních infekcí prováděných na savcích

zahrnujících laboratorní myši, kozy domácí, pískomily, potkany, morčata, křečky a prasata ukázalo, že *C. baileyi* je infekční pouze pro ptáky (Current et al. 1986, Lindsay et al. 1986a, Lindsay et al. 1986b, Lindsay et al. 1987).

Vysoká mortalita způsobená infekcí dýchacího traktu parazitem *C. baileyi* byla zaznamenána u brojlerových kuřat (Lindsay et Blagburn 1990). Infekce ve spojivkách byla lokalizována přímo ve spojivkovém vaku. Ledviny po infekci *C. baileyi* byly výrazně oteklé a zapálené. Na povrchu byly viditelné močové krystaly a přítomnost parazita byla potvrzena na histologickém vyšetření. Studie rovněž ukázala subakutní intersticiální nefritidu, akutní zánět močové trubice, kdy lokalizace parazitů byla na povrchu epiteliálních buněk ledvin (Abbassi et al. 1999).

Fylogenetická analýza: *Cryptosporidium baileyi* na základě analýzy částí genů SSU rRNA, aktin a COWP se řadí mezi střevní kryptosporidie, blízce příbuzné *Cryptosporidium* avian genotyp I a II (Meireles et al. 2006) a *C. avium* (Holubová et al. 2016), ale sekvence genu HSP70 klastrují spíše k žaludečním kryptosporidiím (Xiao et al. 2002).

Morfologie a morfometrie oocyst: Životaschopné oocysty mají velikost 6,0–7,5 μm 4,8 μm × 5,7 μm s poměrem délky a šířky 1,05–1,79 μm (Lindsay et al. 1989).

Věková specifita: Prepatentní perioda je výrazně kratší a patentní perioda výrazně delší u juvenilních ptáků. Délka prepatentní periody u kuřat různého stáří je 3 dny, patentní perioda je od 4 do 24 dnů. Věková variabilita u *C. baileyi* byla pozorována u kuřat infikovaných ve věku 1 a 9 týdnů. Kuřata infikovaná v 1. týdnu života vylučovala 3× více oocyst než kuřata infikovaná v 9 týdnech života (Sréter et al. 1995, Sréter et Varga 2000).

Zoonotický potenciál: V roce 1991 Ditrich et al. (1991) popsali přirozenou infekci *C. baileyi* u imunodeficitního pacienta. Nicméně s ohledem na absenci genotypizace nelze z pohledu dnešních znalostí spolehlivě potvrdit, že se skutečně jednalo o druh *C. baileyi*. V Polsku byl zaznamenán případ nezhoubného nádoru v plicích 51 leté ženy. Mikroskopické a molekulární vyšetření tkáně a tekutiny z okolí nádoru prokázalo přítomnost oocyst, respektive specifické DNA *C. baileyi* (Kopacz et al. 2020).

- ***Cryptosporidium galli* Pavlásek, 1999**

Hostitelská specifita: Jedná se o žaludeční kryptosporidii infikující proventrikulus svého ptačího hostitele. Hostitelské spektrum je velmi široké, infikuje ptáky z řádů hrabavých (Galliformes), papoušků (Psittaciformes), pěvců (Passeriformes), plameňáků (Phoenicopteriformes) a zoborožců (Bucerotiformes) (Mathis et al. 1999, Pavlásek 2001, Ryan et al. 2003a, Ng et al. 2006).

Lokalizace a patogenita: Infekce je doprovázena klinickými příznaky a vysokou mortalitou. V mnoha případech uhynuli infikovaní ptáci po nástupu průjmů (Blagburn et al. 1990, Mathis et al. 1999, Morgan et al. 2001, Pavlásek 2001). Histologické nálezy ukazují hyperplazii epitelových buněk proventrikulárních žláz s velkým počtem vývojových stádií kryptosporidií, které jsou soustředěny na povrchu epiteliálních buněk (Morgan et al. 2001). Prepatentní ani patentní perioda není známa.

Fylogenetická analýza: Na základě fylogenetických analýz spadá *C. galli* do větve žaludečních kryptosporidií blízce příbuzných se skupinou genotypů *Cryptosporidium* sp. finch genotyp I–III (Morgan et al. 2001) a *Cryptosporidium* sp. avian genotyp VI (Chelladurai et al. 2016).

Morfologie a morfometrie oocyst: Oocysty jsou elipsovitého tvaru. Uvnitř oocysty je sférické zbytkové tělísko o velikosti 3,6–4,0 μm, 3 zbytková granula a 4 sporozoiti ve tvaru banánů. Velikost oocyst je 8,0–8,5 μm (průměr 8,25 μm) × 6,2–6,4 μm (průmer 6,3 μm) s poměrem délky a šířky 1,30 μm (Mathis et al. 1999, Ryan et al. 2003b).

Věková specifita: Pomocí experimentální studie bylo prokázáno, že 9denní kuřata se nakazí *C. galli*, kdežto 40denní kuřata nikoli (Ryan et al. 2003b).

Zoonotický potenciál: *Cryptosporidium galli* není infekční pro člověka.

- ***Cryptosporidium avium* Holubová, Sak, Horčičková, Hlásková, Květoňová, Menchaca, McEvoy & Kváč, 2016**

Dříve známo jako *Cryptosporidium* sp. avian genotyp V. Tento druh byl popsán jako platný druh rodu *Cryptosporidium* v rámci této disertační práce (**příloha II**).

- ***Cryptosporidium proventriculi* Holubová, Zikmundová, Limpouchová, Sak, Konečný, Hlásková, Rajský, Kopacz, McEvoy & Kváč, 2019**

Dříve známo jako *Cryptosporidium* sp. avian genotyp III. Tento druh byl popsán jako platný druh rodu *Cryptosporidium* v rámci této disertační práce (**příloha V**).

- ***Cryptosporidium ornithophilus* Holubová, Tůmová, Sak, Hejzlarová, Konečný, John McEvoy & Kváč, 2020**

Dříve známo jako *Cryptosporidium* sp. avian genotyp II. Tento druh byl popsán jako platný druh rodu *Cryptosporidium* v rámci této disertační práce (**příloha VI**).

- ***Cryptosporidium* sp. avian genotyp I**

Tento genotyp byl nalezen u kanára divokého (*Serinus canaria*) (Ng et al. 2006) a páva korunkatého (*Pavo cristatus*) (Nakamura et al. 2009). Jedná se o střevní kryptosporidii, která je fylogeneticky příbuzná s *C. avium, C. baileyi* a *Cryptosporidium* sp. avian genotyp II. Žádné další bližší biologické nebo molekulární charakteristiky nejsou známy.

- ***Cryptosporidium* sp. avian genotyp IV**

Tento genotyp je fylogeneticky nejpříbuznější k *C. galli* a na základě fylogenetických analýz se předpokládá, že se jedná o kryptosporidii s lokalizací v žaludku. Oocysty jsou velikostně identické s oocystami *C. galli* (8,3 × 6,3 μm; Ryan et al. 2003a).

- ***Cryptosporidium* sp. avian genotyp VI**

Více informací o tomto genotypu je uvedeno v přiložené publikaci, která vznikla za spolupráce s kolegy North Dakota State University, ND, USA (**příloha I**).

- ***Cryptosporidium* sp. avian genotyp VII–IX**

Tyto tři genotypy byly nalezeny v Německu u krocanů a kuřat. Dle fylogenetického zařazení se jedná o střevní kryptosporidie (Didier et Weiss 2011). Žádné další bližší biologické nebo molekulární charakteristiky nejsou známy.

- ***Cryptosporidium* sp. Eurasian woodcock genotyp**

Oocysty *Cryptosporidium* Eurasian woodcock genotyp (8,5 × 6,4 μm) se velikostně podobají *C. galli*, nicméně strukturou, tvarem a velikostí granulí v oocystách se liší. Histologické vyšetření zažívacího traktu přirozeně infikované sluky prokázalo přítomnost vývojových stádií pouze v proventrikulu. Genotyp byl odlišen od ostatních zástupců rodu *Cryptosporidium* na genech kódujích SSU, GP60, aktin a HSP70 (Ryan et al. 2003a, Ng et al. 2006).

- ***Cryptosporidium* sp. duck genotyp I, II a IIb**

Cryptosporidium duck genotyp byl detekován u kachny černé (*Anas rubripes*) a husy kanadské (*Branta canadensis*) (Morgan et al. 2001, Jellison et al. 2004, Zhou et al. 2004). Fylogenetická analýza SSU ukázala jeho blízkou příbuznost s *Cryptosporidium* sp. goose genotyp I a II. *Cryptosporidium* duck genotyp IIb byl nalezen u vodních ptáků v severním Španělsku (Cano et al. 2016). Žádné další bližší biologické nebo molekulární charakteristiky nejsou známy.

- ***Cryptosporidium* sp. goose genotyp I–IV, Id**

Cryptosporidium goose genotyp I a II byly nejčastěji identifikovány ve vzorcích trusu husy kanadské, což naznačuje úzkou vazbu na daného hostitele (Morgan et al. 2001). Fylogenetická analýza prokázala podobnost s *Cryptosporidium* sp. duck genotyp I (Zhou et al. 2004). *Cryptosporidium* goose genotyp Id byl nalezen u vodních ptáků v severním Španělsku (Cano et al. 2016). Žádné další bližší biologické nebo molekulární charakteristiky nejsou známy.

- ***Cryptosporidium* sp. finch genotyp I–III**

Tyto genotypy byly detekovány u amadiny Gouldové (*Erythrura gouldiae*). Fylogeneticky jsou tyto genotypy úzce příbuzné *C. galli* a patří mezi žaludeční kryptosporidie (Morgan et al. 2000c).

- **Další genotypy kryptosporidií ptáků**

Cryptosporidium genotyp označený YS-2017 byl nalezen u puštíka hnědého (*Strix leptogrammica*) a puštíka tečkovaného (*Strix seloputo*) v Japonsku. Je fylogeneticky blízce příbuzný s *C. avium* a *Cryptosporidium* sp. avian genotyp II, jedná se tedy o

střevní kryptosporidii. Velikost oocyst tohoto genotypu je 5,40 × 4,13 μm. Dle záznamů způsobuje tento genotyp klinické onemocnění charakterizované průjmy, dehydratací a zvracením (Makino et al. 2018). Gomes et al. (2012) nalezli u kanára divokého (*Serinus canarius*) dosud nepopsané genotypy, které byly pracovně nazvány canary I a II (GU074388-89) a další nový genotyp Jawa sparrow (GU074390) u chůvičky japonské (*Lonchura striata domestica*). Všechny tyto genotypy patří mezi střevní kryptosporidie a jsou fylogeneticky příbuzné s *C. parvum* a *C. meleagridis*.

4. KOMENTÁŘ K VÝSLEDKŮM

Veškeré získané výsledky (obrázky, grafy, tabulky), použitý materiál a metody je možné nalézt v přiložených publikacích.

4.1Výskyt a diverzita kryptosporidií

V rámci doktorského studia bylo získáno a vyšetřeno 4740 vzorků trusu ptáků z 25 řádů a 176 rodů z 9 zemí (Tabulka 4). Z toho 2928 vzorků pocházelo z České republiky, 301 ze Slovenska, 732 z Chorvatska, 25 z Polska, 90 z Jihoafrické republiky, 238 z Nového Zélandu, 11 ze Spojených arabských emirátů, 345 z Alžírska a 70 z USA. Celkový počet pozitivních vzorků ptáků na kryptosporidie byl 255 (5,4 %). Tento výsledek odpovídá průměrné promořenosti volně žijících ptáků 3,96 % (1945/49129, 95 % CI: 3,79–4,13; Wang et al. 2021).

Tabulka 4. Počet vyšetřených a na kryptosporidie pozitivních ptáků v rámci všech provedených studií, které jsou podkladem pro tuto práci.

Řád	Rod	Počet vyšetřených	Počet pozitivních
Accipitriformes	*Accipiter* (2), *Aquila* (1), *Buteo* (3), *Cardinalis* (1), *Circaetus* (1), *Circus* (2), *Haliaeetus* (5), *Milvus* (1), *Neophron* (1), *Pernis* (2)	19	0
Anseriformes	*Alopochen* (1), *Anas* (584), *Anser* (514), *Branta* (2), *Cairina* (2), *Cyanochen* (1), *Cygnus* (25), *Mareca* (1), *Somateria* (1) *Spatula* (2), *Tadorna* (1)	1134	118
Apodiformes	*Apus* (3)	3	0
Apterygiformes	*Apteryx* (23)	23	0
Bucerotiformes	*Bycanistes* (1)	1	0
Casuariiformes	*Dromaius* (8)	8	0
Ciconiiformes	*Ciconia* (33), *Leptoptilos* (2)	35	0
Coliiformes	*Colius* (5)	5	0
Columbiformes	*Columba* (793), *Gallicolumba* (1), *Chalcophaps* (1), *Ocyphaps* (1), *Streptopelia* (5)	801	20
Coraciiformes	*Coracias* (1), *Dacelo* (1), *Alcedo* (29)	31	0
Cuculiformes	*Centropus* (1)	1	0
Falconiformes	*Falco* (9)	9	1
Galliformes	*Bonasa* (1), *Catreus* (2), *Colinus* (1), *Coturnix* (13), *Crossoptilon* (1), *Gallus* (291), *Chrysolophus* (1), *Lophophorus* (1), *Lophura* (1), *Meleagris* (39), *Numida* (2), *Pauxi* (2), *Pavo* (19) *Phasianus* (18), *Tetrao* (18), *Tragopan* (1)	411	29
Gruiformes	*Anthropoides* (1), *Fulica* (1), *Balearica* (4), *Grus* (1), *Porphyrio* (62), *Porzana* (1)	70	0
Charadriiformes	*Actitis* (6), *Burhinus* (1), *Calidris* (42) *Gallinago* (4), *Charadrius* (4), *Chroicocephalus* (64), *Tringa* (6)	127	0

Tabulka 4. Počet vyšetřených a na kryptosporidie pozitivních ptáků v rámci všech provedených studií, které jsou podkladem pro tuto práci (pokračování).

Řád	Rod	Počet vyšetřených	Počet pozitivních
Musophagiformes	*Tauraco* (1)	1	0
Passeriformes	*Acridotheres* (1), *Acrocephalus* (294), *Aegithalos* (1), *Agelaius* (70), Amadina (9), Andropadus (2), Campanula (2), *Caprimulgus* (33), *Carduelis* (70), *Cettia* (11), *Coccothraustes* (1), *Corvus* (9), *Cossypha* (2), *Creatophora* (1), *Crithagra* (2), *Cyanecula* (19), *Cyanistes* (28), *Cyanomitra* (2), *Erithacus* (28), *Erythrura* (15), *Euplectes* (1), *Fringilla* (7), *Garrulus* (2), *Hippolais* (3), *Hirundo* (158), *Chloris* (1), *Lagonosticta* (1), *Lamprotornis* (3), *Laniarius* (1), *Lanius* (2), *Locustella* (8), *Lonchura* (5), *Motacilla* (7), *Muscicapa* (2), *Oenanthe* (1), *Onychognathus* (1), *Paroaria* (3), *Parus* (143), *Passer* (14), *Phoenicurus* (1) *Phylloscopus* (23), *Plocepasser* (5), *Ploceus* (14) *Prinia* (2), *Prunella* (11), *Pycnonotus* (6), *Pyrrhula* (1), *Riparia* (3), *Saxicola* (3), *Serinus* (63), *Sigelus* (2), *Sitta* (11), *Spinus* (2), *Sturnus* (7), *Sylvia* (96), *Sylvietta* (1), *Taeniopygia* (13), *Tragopan* (3), *Turdus* (12), *Uraeginthus* (2), *Urolestes* (1), *Zosterops* (5)	1250	44
Pelecaniformes	*Bubulcus* (1), *Ixobrychus* (1), *Pelecanus* (1), *Plegadis* (1), *Scopus* (1)	5	0
Phoenicopteriformes	*Phoenicopterus* (2)	2	0
Piciformes	*Dryocopus* (1), *Indicator* (1), *Lybius* (1), *Pogoniulus* (1), *Trachyphonus* (1)	5	0
Psittaciformes	*Cyanoramphus* (28), *Psittacula* (8), *Psittacus* (18), *Agapornis* (52), *Alisterus* (8), *Amazona* (5), *Aprosmictus* (2), *ara* (7), *aratinga* (1), *arini* (3), *Barnardius* (2), *Cacatua* (5), *Deroptyus* (2), *Eclectus* (9), *Lathamus* (1), *Melopsittacus* (101), *Neophema* (2), *Neopsephotus* (1), *Nestor* (31), *Nymphicus* (130), *Platycercus* (16), *Poicephalus* (44), *Polytelis* (4), *Psephotus* (7), *Pyrrhura* (2)	489	27
Sphenisciformes	*Spheniscus* (1)	1	0
Strigiformes	*Aegolius* (1), *Asio* (3), *Athene* (1), *Bubo* (5), *Glaucidium* (2), *Ninox* (6), *Otus* (15), *Surnia* (1), *Tyto* (4)	38	0
Struthioniformes	*Struthio* (271)	271	16
Celkem		**4740**	**255**

Procento infikovaných ptáků se napříč různými studiemi a věkovými kategoriemi pohybuje od 0 do 100 % (Curtiss et al. 2015, Iijima et al. 2018, Wang et al. 2021). Velký rozptyl jak v publikovaných, tak našich výsledcích může být ovlivněn řadou faktorů. Častou příčinou může být způsob výpočtu prevalence z různě velkých skupin vyšetřovaných jedinců. Poměrně často jsou publikovány výsledky, které jsou

získány pouze z malého vzorku jedinců, někdy pouze z jednoho jedince (Nakagun et al. 2017, Oliveira et al. 2017b, Seixas et al. 2019). Také výpočet prevalence z již předem vybrané skupiny, například ptáků trpících klinickým onemocněním, výrazným způsobem zkresluje výsledky (Curtiss et al. 2015, Makino et al. 2018). Této chybě jsme se v naší práci snažili vyhnout, i když z některých zástupců se nám nepodařilo získat reprezentativní počet vzorků.

Druhým, velmi důležitým faktorem je hostitelská specifita jednotlivých druhů a genotypů kryptosporidií. V rámci námi provedených studií jsme nejčastěji detekovali *C. baileyi* (65/4740; 1,4 %), *C. proventriculi* (35/4740; 0,7 %) a *Cryptosporidium* sp. goose genotyp Id (33/4740; 0,7 %). Naopak nejméně často byl detekován *Cryptosporidium* sp. avian genotyp I (2/4740; 0,04 %). Přehled všech nalezených druhů a genotypů je přehledně popsán v Tabulce 5. Zatímco druh *C. baileyi*, který byl v rámci našich studií detekován u 15 druhů ptáků patřících do 13 rodů v rámci 9 čeledí ze 7 řádů (hrabavých, měkkozobých, papoušků, pěvců, pštrosů, vrubozobých a sokolů) a celosvětově dále také u dravců a sov (Ng et al. 2006, Nakamura et al. 2009, Qi et al. 2011, Seva Ada et al. 2011, Baroudi et al. 2013, Li et al. 2015a, Máca et Pavlásek 2016, Wang et al. 2021), *Cryptosporidium* sp. avian genotyp I se zdá být hostitelsky adaptován pouze na kanára divokého (*Serinus canaria*), což se shoduje jak s našimi výsledky práce, tak se studiemi ve světě (Ng et al. 2006, Camargo et al. 2018). Obdobně tomu je u *Cryptosporidium* avian genotypu VI, který byl nalezen v rámci této práce pouze u vlhovců červenokřídlých (*Agelaius phoeniceus*) (Chelladurai et al. 2016). U kachny divoké (*Anas platyrhynchos*) jsme popsali izolát, který nebyl doposud popsán a v brzké době bude opublikován jako nový ptačí genotyp.

Tabulka 5. Přehled nalezených druhů a genotypů kryptosporidií u vyšetřených ptáků.

Hostitel (vědecké jméno)	Země	Druh/genotyp (počet záchytů)	Publikace
amada Gouldové (*Erythrura gouldiae*)	POL	*C. galli* (2)	Příloha V
amadina páskovaná (*Amadina fasciata*)	ZAF	*C. galli* (1)	Dosud nepublikováno
andulka vlnkovaná (*Melopsittacus undulatus*)	CZE	*C. avium* (1); *C. baileyi* (2)	Příloha V
čížek lesní (*Spinus spinus*)	CZE	*C. baileyi* (1)	Dosud nepublikováno
čížek mexický (*Spinus psaltria*)	CZE POL	*C. baileyi* (1) *C. galli* (2)	Příloha V Příloha V
drozd zpěvný (*Turdus philomelos*)	CZE	*C. galli* (1)	Dosud nepublikováno
holub domácí (*Columba livia*)	CZE	*C. meleagridis* (6); *C. muris* (2); *C. ornithophilus* (2); *C. parvum* (4); *C. baileyi* (2); *C. galli* (1)	Dosud nepublikováno
	SVK	*C. andersoni* (2); *C. meleagridis* (1)	Dosud nepublikováno
husa bílá (*Anser anser* f. *domestica*)	CZE	*C. parvum* (1); *C. baileyi* (2); goose genotyp Id (19); duck genotyp (6)	Dosud nepublikováno
husa velká (*Anser anser*)	DZA	*C. meleagridis* (1)	Příloha III
	CZE	*C. parvum* (1); goose genotyp Id (14), goose genotyp (5)	Dosud nepublikováno
kachna divoká (*Anas platyrhynchos*)	DZA	*C. baileyi* (1)	Přílohy II, IV a V
	CZE	*C. proventriculi* (14); *C. avium* (6); *C. baileyi* (13); duck genotyp (14); nový izolát (16)	
kachna domácí (*Anas platyrhynchos* f. *domestica*)	CZE	*C. baileyi* (5)	Dosud nepublikováno
kakariki rudočelý (*Cyanoramphus novaezelandiae*)	CZE	*C. avium* (2)	Přílohy II a V
kanár divoký (*Serinus canaria*)	CZE	avian genotyp I (2)	Příloha V
korela chocholatá (*Nymphicus hollandicus*)	CZE	*C. baileyi* (2); *C. proventriculi* (13)	Příloha V
	SVK	*C. proventriculi* (2)	Příloha V
krůta domácí (*Meleagris gallopavo* f. *domestica*)	DZA	*C. meleagridis* (2)	Příloha III
kur domácí (*Gallus gallus* f. *domesticus*)	DZA	*C. baileyi* (12); *C. meleagridis* (11)	Příloha III
	CZE	*C. suis* (1)	Dosud nepublikováno
mlynařík dlouhoocasý (*Aegithalos caudatus*)	CZE	*C. baileyi* (1)	Dosud nepublikováno
papoušek koňžský (*Poicephalus gulielmi*)	CZE	*C. proventriculi* (2)	Příloha V
Papoušík růžohrdlý (*Agapornis roseicollis*)	CZE	*C. proventriculi* (2)	Příloha V
	SVK	*C. proventriculi* (1)	Příloha V
páv korunkatý (*Pavo cristatus*)	POL	*C. baileyi* (3)	Dosud nepublikováno

Tabulka 5. Přehled nalezených druhů a genotypů kryptosporidií u vyšetřených ptáků (pokračování).

Hostitel (vědecké jméno)	Země	Druh/genotyp (počet záchytů)	Publikace
pěnkava obecná (*Fringilla coelebs*)	CZE	*C. galli* (1)	Dosud nepublikováno
pštros dvouprstý (*Struthio camelus*)	DZA	*C. baileyi* (4)	Příloha III
	CZE	*C. ornithophilus* (7*); C. ubiquitum* XIIa (5)	Příloha VI
sokol stěhovavý (*Falco peregrinus*)	ARE	*C. baileyi* (1)	Dosud nepublikováno
sýkora koňadra (*Parus major*)	CZE	*C. baileyi* (11); *C. galli* (5)	Dosud nepublikováno
sýkora modřinka (*Cyanistes caeruleus*)	CZE	*C. baileyi* (2)	Dosud nepublikováno
vlhovec červenokřídlý (*Agelaius phoeniceus*)	USA	avian genotyp VI (11); *C. galli* (1)	Příloha I
zebřička pestrá (*Taeniopygia guttata*)	POL	*C. galli* (1)	Příloha V

ARE: Spojené arabské emiráty; **CZE:** Česká republika; **DZA:** Alžírsko; **POL:** Polsko; **SVK:** Slovensko; **USA:** Spojené státy americké

4.2 Biologická a molekulární charakterizace nových druhů a genotypů kryptosporidií

4.2.1 Morfologie a morfometrie oocyst ptačích kryptosporidií

Ačkoli morfologie a morfometrie oocyst je jednou ze základních podmínek pro popis nového druhu, nejsou tyto údaje spolehlivým diagnostickým znakem a pro identifikaci druhu a genotypu kryptosporidií a vždy je nezbytná molekulární charakterizace. Mikroskopicky je od sebe možné odlišit druhy parazitující v žaludku, které mají obecně větší, oválné oocysty, od druhů s vývojem ve střevě, které mají menší okrouhlé oocysty. Také v rámci jednotlivých fylogenetických linií existují významné rozdíly. Oocysty *C. galli* (žaludeční druh) jsou mnohem větší než oocysty střevního druhu *C. ornithophilus* (Tabulka 6), ale v rámci střevních kryptosporidií nelze od sebe odlišit oocysty *C. ornithophilus*, *C. avium* a *C. baileyi* (Holubová et al. 2020). *Cryptosporidium meleagridis* má morfometricky identické oocysty s druhem *C. parvum*, za který bylo často v minulosti zaměňováno a který byl v několika případech u ptáků detekován (Graczyk et al. 1998, Champliaud et al. 1998, Sréter et Varga 2000, Zhou et al. 2004, Zylan et al. 2008, Mcevoy et Giddings 2009, Nakamura et al. 2009, Gomes et al. 2012, Helmy et al. 2017, Oliveira et al. 2017b). Přestože je velikost oocyst *C. meleagridis* výrazně menší než u ostatních ptačích kryptosporidií parazitujících ve střevě, skutečné využití rozdílů mezi druhy při jejich

diferenciaci je velmi omezené. Obdobně je tomu i u v rámci linie žaludečních kryptosporidií. Oocysty *C. galli* mohou být zaměněny s oocystami *Cryptosporidium* avian genotyp IV a *Cryptosporidium* Eurasian woodcock genotyp. Přestože všechny zmíněné kryptosporidie mají oocysty větší než *C. proventriculi*, rozdíl ve velikosti není prakticky využitelný pro rozlišení pomocí rutinní světelné mikroskopie.

Tabulka 6. Přehled ptačích druhů kryptosporidií, u kterých je známa velikost oocyst.

Druh	Velikost oocyst (µm)			Hostitel	Citace
	Délka		Šířka		
*C. avium**	6,3 (5,3–6,9)	×	4,9 (4,3–5,5)	kakariki rudočelý	Holubová et al. (2016)
C. baileyi	6,3 (6,0–7,2)	×	5,2 (4,8–5,4)	kur domácí	Current et al. (1986)
C. galli	8,25 (8,0–8,5)	×	6,3 (6,2–6,4)	kur domácí	Pavlásek (1999)
C. meleagridis	5,2 (4,5–6,0)	×	4,6 (4,2–5,3)	krůta domácí	Slavin (1955)
*C. ornithophilus**	6,1 (5,2–6,8)	×	5,2 (4,7–5,5)	pštros dvouprstý	Holubová et al. (2020)
*C. proventriculi**	7,35 (6,7–8,4)	×	5,7 (5,1–6,3)	korela chocholatá	Holubová et al. (2019)
Cryptosporidium avian genotyp IV	8,25	×	6,3	kruhoočko japonské	Ryan et al. (2003a)
Cryptosporidium Eurasian woodcock genotyp	8,5	×	6,4	sluka lesní	Ryan et al. (2003a)

* druhy popsané v rámci disertační práce

Cryptosporidium avium

Oocysty *C. avium* pocházející z přirozeně infikovaných papoušků (kakariki rudočelý) byly morfometricky shodné s oocystami, které byly získány z experimentálně infikovaných slepic a které měřily 5,30–6,90 µm (průměr = 6,26 µm) × 4,30–5,50 µm (průměr = 4,86 µm) a indexu tvaru 1,29 (1,14–1,47). Oocysty *C. avium* nelze morfometricky odlišit od oocyst dalších ptačích druhů a genotypů kryptosporidií jako například *C. baileyi* a *C. ornithophilus* (Meireles et al. 2006, Ng et al. 2006, Qi et al. 2011, Holubová et al. 2016). Podrobný popis a fotodokumentace oocyst *C. avium* je v **příloze II**.

Cryptosporidium proventriculi

Oocysty *C. proventriculi* jsou z hostitele vylučovány vysporulované a obsahují 4 sporozoity a reziduální tělísko. Velikost oocyst získaných z přirozeně infikovaných korel [6,70–8,40 μm (průměr ± SD = 7,35 ± 0,41 μm) × 5,10–6,30 μm (průměr ± SD = 5,70 ± 0,32 μm) s poměrem mezi délkou a šířkou 1,08–1,41 (průměr ± SD = 1,23 ± 0,11)] se nelišila od oocyst získaných z experimentálně infikovaných korel, u kterých měřily oocysty 6,60–8,40 μm (průměr ± SD = 7,37 ± 0,44 μm) × 5,00–6,40 μm (průměr ± SD = 5,80 ± 0,35 μm) s poměrem mezi délkou a šířkou 1,06–1,43 (průměr ± SD = 1,25 ± 0,10). Oocysty *C. proventriculi* jsou větší než oocysty *C. avium* a *C. baileyi* ($P < 0,001$) a nepatrně menší než oocysty *C. galli*. Podrobný popis a fotodokumentace oocyst *C. proventriculi* je **v příloze V**.

Cryptosporidium ornithophilus

Oocysty z přirozeně infikovaných pštrosů měřily 5,24–6,77 μm (průměr ± SD = 6,13 ± 0,35 μm) × 4,68–5,5 μm (průměr ± SD = 5,15 ± 0,24 μm) s poměrem délky k šířce 1,06–1,36 (průměr ± SD = 1,19 ± 0,08) a shodovaly se velikostí s oocystami získanými z experimentálně infikovaných kuřat, housat a korel. Velikost oocyst *C. ornithophilus* získaných v rámci disertační práce (6,1×5,1 μm) byla srovnatelná s výsledky uveřejněnými ve studiích Santos et al. (2004) a Meireles et al. (2006), kde oocysty měřily 6,0×4,8 μm. Podrobný popis a fotodokumentace oocyst *C. ornithophilus* je **v příloze VI**.

4.2.2 Hostitelská a tkáňová specifita ptačích kryptosporidií

Studie o průběhu kryptosporidiových infekcí a hostitelské a tkáňové specifitě jsou často chybějící části biologické charakteristiky kryptosporidií, a to nejenom u druhů infikujících ptáky (Vetterling et al. 1971, Iseki 1979, Pavlásek et al. 1995, Fayer et al. 2001, Ryan et al. 2003b, Fayer et al. 2005, Sitja-Bobadilla et al. 2006, Fayer et al. 2008, Jirků et al. 2008, Power et Ryan 2008, Ryan et al. 2008, Fayer et Santín 2009, Fayer et al. 2010, Robinson et al. 2010, Traversa 2010, Elwin et al. 2012, Kváč et al. 2013a, Kváč et al. 2014a, Li et al. 2015b, Ryan et Hijjawi 2015, Holubová et al. 2016, Chelladurai et al. 2016, Ježková et al. 2016, Zahedi et al. 2017, Čondlová et al. 2018, Kváč et al. 2018, Holubová et al. 2019, Horčičková et al. 2019, Ježková et al. 2021b, Zahedi et al. 2021).

Morfologie a morfometrie dalších stadií životního cyklu a variabilita v rámci životního cyklu (např. další vývojová stádia – merogonie), stejně jako určení

predilekčního místa infekce může poskytnout cenné informace pro odlišení jednotlivých druhů (Holubová et al. 2020, Ježková et al. 2021a). Tento přístup však vyžaduje velké úsilí zahrnující podrobné zkoumání každého hostitele z hlediska infikovaných tkání a následnou časově a finančně náročnou analýzu pomocí různých typů barvení, histologické zpracování tkání a elektronovou mikroskopii. Zmíněný přístup je pro běžnou diagnostiku nepraktický a dalo by se říct i nepoužitelný, ale pro popis nových druhů je tento postup nezbytný (Kváč et al. 2014a, Ježková et al. 2021b).

Výsledky řady studií provedených nejen na ptácích ukázaly, že jednotlivé druhy, a samozřejmě i genotypy kryptosporidií jsou charakterizovány úzkou tkáňovou specifitou. Vývoj příslušného druhu kryptosporidie je většinou vázán na jedno konkrétní predilekční místo infekce (Lindsay et al. 2000, Ryan et al. 2008, Kváč et al. 2014a, Kváč et al. 2018, Holubová et al. 2020). Znalost místa infekce spolu s morfologickou charakterizací oocyst může být velmi užitečná při determinaci druhu/genotypu. Nicméně lze najít řadu případů, kdy se morfologie oocyst a lokalizace infekce mezi jednotlivými druhy překrývá. Příkladem může být *C. ornithosphilus*, které se vyskytuje ve slepém střevě stejně jako *C. avium* (Holubová et al. 2016, Holubová et al. 2020). V tenkém střevě některých hlodavců, konkrétně v jejunu a ileu, byly lokalizovány druhy *C. ditrichi* a *C. alticolis* (Čondlová et al. 2018, Horčičková et al. 2019). V tenkém střevě prasat lze nalézt *C. scrofarum* a *C. parvum* (Kváč et al. 2013a, Li et al. 2013). V tlustém střevě potkanů se vyskytuje nejen *C. occultus,* ale výjimečně také *C. meleagridis* (Kimura et al. 2007, Kváč et al. 2013a, Kváč et al. 2018).

Na základě našich experimentů jsme mohli určit tkáňovou specifitu u námi popsaných druhů. *Cryptosporidium ornithophilus* infikuje slepé a tlusté střevo, a Fabriciovu burzu, *C. avium* bylo nalezeno v tenkém a slepém střevě, ale také ledvinách a močovodech a *C. proventriculi* bylo nalezeno v proventrikulu a ventrikulu.

Další, neméně významnou biologickou vlastností jednotlivých druhů a genotypů kryptosporidií je jejich hostitelská specifita (Sréter et al. 2000a, Li et al. 2016, Holubová et al. 2018, Widmer et al. 2020). Na základě experimentálních infekcí, které jsou podrobně rozepsané v publikacích v **příloze II.** a **IV.** bylo prokázáno, že *C. avium* získané z kakarikiho rudočelého patřícího do řádu papouškovitých, je

infekční pro kur domácí (*Gallus gallus* f. *domestica*) z řádu hrabavých, kachnu domácí (*Anas platyrhynchos* f. *domestica*) patřící do řádu vrubozobých, ale překvapivě neinfekční pro bažanta obecného (*Phasianus colchicus*). Vnímavost kura domácího k infekci *C. avium* byla popsána již v roce 2014 u brojlerových kuřat v Číně (Wang et al. 2014). Oproti *C. meleagridis*, u kterého byla popsána infektivita pro savce včetně myší, potkanů, králíků, skotu a člověka (Pedraza-Diaz et al. 2001, Cama et al. 2003, Darabus et Olariu 2003, Xiao et al. 2004a, Elwin et al. 2012), nebyla infekce *C. avium* popsána u jiných než ptačích hostitelů. V případě *C. proventriculi* jsme na základě experimentů zjistili, že tento druh je infekční pro korelu chocholatou z řádu papoušků, nikoliv však andulku vlnkovanou, která také patří mezi papoušky. Dále *C. proventriculi*, které bylo detekováno u řady zástupců řádu papoušků, pěvců, šplhavců, a vrubozobých (Nakamura et Meireles 2015, Cano et al. 2016, Ferrari et al. 2018, Silva Novaes et al. 2018) není infekční pro kuřata a SCID myši (**příloha V**). Díky provedeným experimentům jsme dále zjistili, že *C. ornithophilus* není infekční pro savce (laboratorní myši s různým stupněm imunodeficience), ale naopak je infekční pro husy a korely. Na rozdíl od studií Meireles et al. (2006), Santos et al. (2005), se nám podařilo experimentálně přenést infekci *C. ornithophilus* na kuřata. Výsledky naší práce a dalších studií ukázaly, že *C. ornithophilus* infikuje celou řadu různých ptačích hostitelů různých řádů ptáků (Meireles et al. 2006, Ng et al. 2006, Nakamura et al. 2009, Seva Ada et al. 2011, Nguyen et al. 2013). Tato širší hostitelská specifita je obdobná jako byla pozorována u fylogeneticky příbuzného druhu *C. avium*, ale podstatně menší, než je u *C. baileyi*, druhu, který je infekční pro většinu ptáků (Current et al. 1986, Bermudez et al. 1988, Jellison et al. 2004, Kimura et al. 2004, Molina-Lopez et al. 2010, Curtiss et al. 2015, Holubová et al. 2016) (**příloha VI**).

Sledování hostitelské a s tím spojené tkáňové specifity je zatíženo několika potenciálními problémy. Na základě doporučení uvedených v literatuře, ale zejména na základě vlastních zkušeností je třeba přihlížet na stáří, podmínkám skladování a metodám zpracování oocyst, které mohou ovlivňovat jejich infekce schopnost a na imunitní stav a věk hostitele, které mohou významně ovlivnit výsledek experimentu (Fayer 2007, Kváč et al. 2013a, Holubová et al. 2016, Kváč et al. 2016, Holubová et al. 2018). Ideální je používat čerstvé, nanejvýš dva měsíce staré oocysty, které jsou skladovány při teplotě 4–8 °C (Kváč et al. 2018, Horčičková et al. 2019, Ježková et

al. 2021a). Imunosuprimovaní a imunodeficitní jedinci, jsou více vnímaví k infekci kryptosporidií, ale prepatentní perioda může být výrazně prodloužena (Kváč et al. 2011). Tento fenomén je třeba brát v úvahu, aby nedošlo k předčasnému ukončení experimentu (Kváč et al. 2008). Také jedinci, kteří se již v minulosti setkali s kryptosporidiovou infekcí mohou být k následné infekci rezistentní nebo méně vnímaví, což se může projevit nižší intenzitou infekce a délkou prepatentní a patentní periody (Jalovecká et al. 2010). Toto je třeba brát do úvahy, zejména v případech, kdy jsou pro experimentální infekce používána zvířata z konvenčních chovů nebo odchycená ve volné přírodě.

4.2.3 Variabilita věkové specifity ptačích kryptosporidií

Existuje jen málo studií, které se zabývaly přímo věkovou specifitou kryptosporidií ptáků, a proto jsme se na toto téma zaměřili. Mnoho hostitelů vykazuje v průběhu ontogeneze změny v citlivosti vůči infekčním agens. U rodu *Cryptosporidium* byly popsány tři typy vztahů mezi hostitelem a parazitem v závislosti na věku (Kváč et al. 2014c).

1. Vnímavost parazita není závislá na věku hostitele. Příkladem jsou:

- *Cryptosporidium andersoni* – jsou vnímavé všechny věkové kategorie skotu (Kváč et al. 2014c).
- *Cryptosporidium baileyi* – byla úspěšně infikována kuřata v různém věku (Sréter et al. 1995, Sréter et Varga 2000).

2. K infekci jsou vnímaví pouze mladí jedinci.

- Laboratorní myši BALB/c a telata se nakazí *C. parvum* pouze v juvenilním věku (Santín et al. 2004, Fayer et al. 2006, Fayer et al. 2007), dospělí jedinci těchto hostitelů jsou k infekci vnímaví výjimečně.
- *Cryptosporidium galli* je infekční pro 9denní, ale ne pro 40denní kuřata (Pavlásek 2001).

3. K infekci dojde až od určitého věku hostitele.

- *Cryptosporidium scrofarum* je infekční pro prasata starší 5 týdnů (Kváč et al. 2014c).

Průběh infekce, včetně prepatentní a patentní periody, byl do současné doby znám pouze u tří platných ptačích druhů (Slavin 1955, Current et al. 1986, Ryan et al. 2003b). Výsledky našich studií výrazným způsobem rozšířily znalosti o věkové specifitě a průběhu infekce u několika dalších druhů a genotypů kryptosporidií parazitujících u ptáků.

Cryptosporidium avium patří mezi druhy kryptosporidií, u kterých je prokázán přenos parazita na hostitele u všech věkových kategorií vybraných skupin ptáků (**přílohy II a IV**). Prepatentní perioda *C. avium* u kachen domácích (4–6 dní) je podobná prepatentní periodě *C. baileyi* a *C. meleagridis* (4–8 dní; Lindsay et al. 1988, Hornok et al. 1998, Tůmová et al. 2002). Sréter et al. (1995) a Sréter et Varga (2000) popsali vliv věku kuřat na průběh infekce *C. baileyi*. Výsledky jejich studií ukázaly, že prepatentní perioda *C. baileyi* je značně kratší u 9denních než u 1denních kuřat. Současně pozorovali delší patentní periodu u mladších kuřat. Protože druh *C. avium* je fylogeneticky blízce příbuzný právě *C. baileyi*, mohli bychom očekávat obdobný průběh infekce. Tato hypotéza nebyla v naší studii potvrzena. Experimentálně jsme neprokázali vliv věku na délku prepatentní a patentní periody u infekcí způsobených druhem *C. avium*. Přestože ptáci během experimentu vylučovali jen malé množství oocyst, délka infekce trvala po celou dobu experimentu nezávisle na věku zvířat. Lze předpokládat, že námi vyvolané infekce by pokračovaly v chronické formě několik měsíců. Toto tvrzení opíráme o fakt, že kakariki rudočelý, z kterého byl získán izolát pro naši studii vylučoval oocysty *C. avium* více než 5 měsíců (Holubová et al. 2016). Taktéž byla zjištěna infekce vyvolaná *C. avium* u 7letého kakadu inka (*Lophochroa leadbeateri*) (Curtiss et al. 2015), což podporuje tvrzení, že i starší jedinec se může tímto parazitem nakazit.

U *Cryptosporidium ornithophilus* jsme na základě námi provedených experimentů zjistili kratší prepatentní periodu u mladších jedinců (juvenilní jedinci měli prepatentní periodu od 4 do 8 dne po infekci (DPI)) než u dospělých (specifickou DNA začali vylučovat až 8 DPI), kteří mohou být k infekci méně citliví z důvodů již prodělané kryptosporidiové infekce. *Cryptosporidium ornithophilus* se vyskytuje převážně u mladších jedinců, což potvrzuje nález tohoto druhu převážně u kuřat (Wang et al. 2011, Nguyen et al. 2013) a můžeme předpokládat, že nepřítomnost oocyst *C. ornithophilus* u starších pštrosů by mohla být vysvětlena rezistencí

související s věkem. Obdobný fenomén byl popsán u různých hostitelů infikovaných *C. baileyi*, *C. muris* nebo *C. andersoni* (Lindsay et Blagburn 1990, Kváč et al. 2009).

Délka prepatentní periody *C. proventriculi* (6 DPI) byla podobná druhům *C. meleagridis* a *C. baileyi*, které infikují střevo (4–8 dnů; (Lindsay et al. 1988, Rhee et al. 1991, Hornok et al. 1999, Tůmová et al. 2002), a zároveň kratší než 25 dnů, kterou popsal (Pavlásek 2001) u kuřat infikovaných *C. galli*. Na základě mikroskopického, histologického a molekulárního vyšetření gastrointestinálního traktu bylo zjištěno, že SCID myši, andulky vlnkované a kuřata nejsou vnímavá k infekci *C. proventriculi* (**příloha V**).

4.2.4 Patogenita ptačích kryptosporidií

Patogenita kryptosporidií infikujících ptáky, ale také savce, je velmi variabilní. Na základě provedených experimentů jsme zjistili patogenitu u námi popsaných druhů. Žaludeční kryptosporidie u savců nezpůsobují do takové míry poškození žaludku či úhyn hostitele jako například ptačí kryptosporidie *C. galli*, pro kterou je typický výskyt klinického onemocnění s vysokým procentem úmrtnosti (Blagburn et al. 1987, Blagburn et al. 1990, Pavlásek 1999, Morgan et al. 2001, Pavlásek 2001). Mezi známé klinické příznaky *C. galli* patří průjem, apatie, ztráta hmotnosti (Blagburn et al. 1990, Lindsay et al. 1991, Pavlásek 1999, Morgan et al. 2001, Pavlásek 2001, Antunes et al. 2008).

V rámci této práce jsme studovali žaludeční druh *C. proventriculi*, který je jediným popisem žaludeční kryptosporidie, která byla detekována jak ve žláznatém, tak i nežláznatém žaludku. Během našich experimentů jsme nezaznamenali žádné klinické příznaky u infikovaných ptáků (**příloha V**). Makino et al. (2010) popsal chronické zvracení spojené s úbytkem hmotnosti u hrdliček, které byly přirozeně infikované *C. proventriculi*. Histopatologické vyšetření uhynulých hrdliček ukázalo zvětšení proventrikulu a hyperplazii duktálního epitelu proventrikulárních žláz. Rozdíly v naší studii s jinými studiemi si můžeme vysvětlit tím, že zatímco naše pokusy trvaly pouze 30 dní, přirozeně infikovaní ptáci mohli být infikováni i několik měsíců. Podobně byly histopatologické změny a klinické příznaky pozorovány u savců chronicky infikovaných žaludečními druhy *C. proliferans, C. andresoni* nebo *C. muris* (Anderson 1987, Pospischil et al. 1987, Ozkul et Aydin 1994, Esteban et Anderson 1995, Kváč et al. 2016).

U zvířat infikovaných *C. ornithophilus* jsme nezaznamenali žádné klinické příznaky kryptosporidiózy (**příloha VI**), což je v souladu s dalšími studiemi (Meireles et al. 2006, Ng et al. 2006). V některých studiích je spojována infekce kryptosporidiemi pštrosích kuřat s výhřezy kloaky, enteritidy a pankreatitidy (Penrith et al. 1994, Ponce Gordo et al. 2002, Santos et al. 2005). Nicméně v těchto studiích nebyla provedena genotypizace a nelze tedy s jistotou tvrdit, že se jednalo o infekci způsobenou *C. ornithophilus*.

Většina ptáků infikovaných *C. avium*, kteří byli vyšetřeni v rámci této práce, ale i ve světě, včetně experimentálně infikovaných slepic a andulek, nevykazovala žádné klinické příznaky kryptosporidiózy (příloha II; Ng et al. 2006). Curtiss et al. (2015) popsali kryptosporidiózu způsobenou *C. avium* u 7letého kakadu inka (*Lophochroa leadbeateri*), který vykazoval příznaky letargie, anorexii a kloakální prolaps.

4.2.5 Molekulární diagnostika kryptosporidií

Diagnostika kryptosporidií založená na molekulární detekci je ve srovnání s mikroskopickým vyšetřením nejsenzitivnější (Xiao 2010, Kváč et al. 2014c). Udávaná citlivost se nejčastěji pohybuje od 1 až 10 oocyst v analyzovném vzorku (Smith et al. 2006, Thompson et Ash 2016). Hlavní výhodou molekulárních metod je možnost spolehlivě odlišit jednotlivé druhy, genotypy a subtypy kryptosporidií. Další výhodou je možnost izolovat DNA z jakéhokoliv materiálu (Enemark et al. 2002, Xiao et al. 2004a, Xiao et al. 2004b, Smith et al. 2006, Plutzer et Karanis 2007). Ale i molekulární metody mají svá úskalí. Většina všech dnes známých druhů a genotypů je charakterizována na základě odlišnosti v sekvenci SSU. Nicméně použití pouze sekvencí SSU k odvození evolučních vztahů kryptosporidií může vést k chybným závěrům (Li et al. 2014, Stenger et al. 2015, Ježková et al. 2021a). Proto je nutné při fylogenetických analýzách používat i další lokusy, jako jsou geny kódující HSP70, gp60, aktin, COWP nebo TRAP–C1 (Morgan-Ryan et al. 2001, Tang et al. 2016). Zjištění vnitrodruhové variability nebo smíšené infekce vyžaduje **i)** opakované sekvenování produktů PCR, **ii)** klonování produktů PCR s následným sekvenováním nebo **iii)** sekvenování nové generace (Grinberg et al. 2013, Paparini et al. 2015, Stenger et al. 2015). Běžně je používáno Sangerovo sekvenování, ale omezením tohoto přístupu je, že smíšené infekce nejsou obvykle detekovány kvůli nízké intenzitě infekce jednoho nebo více druhů a genotypů kryptosporidií oproti vysoké intenzitě infekce dominantního druhu/genotypu ve vzorku (Grinberg et al. 2013).

Z výše uvedených důvodů byly všechny námi provedené studie založené na multilokusové genotypizaci. Vždy byla provedena genotypizace na SSU lokusu v kombinaci s geny kódujícími aktin a HSP70, případně gp60, COWP nebo TRAP–C1. V případě, že nebylo možné amplifikovat vybraný lokus pomocí běžně používaných sad primerů, byly navrženy vlastní primery (Chelladurai et al. 2016). Pro popis nového druhu kryptosporidie byly vždy sekvenovány minimálně tři geny. Podrobný popis používaných metodik je v přiložených publikacích (**přílohy II**, **V** a **VI**).

4.3 Model kuřecích embryí pro pomnožení oocyst *Cryptosporidium parvum* a *Cryptosporidium baileyi* (příloha VII)

V současné době neexistuje postup, jak dokončit vývojový cyklus kryptosporidií v *in vitro* podmínkách a všechny vědecké týmy ve světě jsou odkázány na získávání parazitů z přirozeně nebo experimentálně infikovaných zvířat (Arrowood 2002). Žádná „úspěšná“ *in vitro* kultivace kryptosporidií nebyla totiž doposud úspěšně zopakována a implementována do běžné laboratorní praxe. V současné době lze k vědeckým účelům využít pouze tři druhy kryptosporidií, *C. parvum*, *C. hominis* a *C. muris*, které jsou komerčně dostupné (Wanyiri et Ward 2006, Vinayak et al. 2015). Vzhledem k tomu, že většina kryptosporidií je úzce hostitelsky specifická, je *in vivo* udržování jednotlivých druhů a genotypů kryptosporidií technicky, ekonomicky a biologicky velmi náročné (Kváč et al. 2013b, Li et al. 2015b). Pokud bychom chtěli pracovat pouze s druhy a genotypy kryptosporidií, které jsou infekční pro člověka (Ortega et Kváč 2013), znamenalo by to získávat oocysty z celé řady různých hostitelů, jako jsou skot, ovce, koně, prasata, králíci, myši, myšice, hraboši, potkani, veverky, ježci nebo drůbež (Kváč et al. 2013a, Elkarim Laatamna et al. 2017, Kváč et al. 2018, Ježková et al. 2021b), což je finančně a technicky velmi náročné. Navíc u těchto hostitelů jsou infekce často asymptomatické s velmi nízkou produkcí oocyst (Kváč et al. 2013a, Kváč et al. 2013b, Němejc et al. 2013, Holubová et al. 2016, Horčičková et al. 2019).

V roce 2014 byl zveřejněn článek o úspěšném pomnožení *C. baileyi* (druh hostitelsky specifický pro ptáky) v embryích kura domácího (Huang et al. 2014). Nezodpovězenou a neotestovanou otázkou zůstávalo, zda jsou ptáci, respektive jejich embrya vnímavá k infekci hostitelsky nespecifickými druhy a genotypy

kryptosporidií. V řadě epidemiologických studií byly u ptáků v malém procentu případů detekovány savčí druhy a genotypy kryptosporidií (Santín et al. 2004, Ng et al. 2006, Zylan et al. 2008, Helmy et al. 2017, Ferrari et al. 2018). Výsledky Huang et al. (2014) nás inspirovali k výzkumu zaměřenému na kultivaci kryptosporidií pomocí kuřecích embryí. Našim cílem bylo zjistit, do jaké míry může infekce ve stádiu embrya zvýšit množení oocyst kryptosporidií u kuřat. Imunita ptáků je v podstatě analogická se systémem savců (Kaiser 2010, Chen et al. 2013). Embryo může být ve vajíčku chráněno pouze pasivní imunitou, která souvisí s mateřskými protilátkami, které jsou přenášeny na potomstvo vaječným žloutkem z imunních matek (Toman 2009). B-buňky se vyvíjejí mezi 7.–14. dnem inkubace a od 15. dne kolonizují sekundární lymfatické orgány. T-buňky, vyvíjející se v brzlíku a které jsou zásadní pro úspěšné zvládnutí kryptosporidiové infekce, opouštějí brzlík 2–3 dny před vylíhnutím (Toman 2009, Jílek 2014). Právě tento fakt spolu s možností získávat násadová vejce od zdravých nosnic dělá z vajec, respektive embryí v nich, potenciálně ideální inkubátor pro kultivaci kryptosporidií.

Pro výzkum jsme použili dva druhy kryptosporidií, a to *C. baileyi* a *C. parvum*, které byly inokulovány do alantoisu devátý den inkubace kuřecích embryí a perorálně do jednodenních kuřat. Výsledky experimentu ukázaly, že:

- Velikost infekční dávky nemá vliv na průběh infekce ani u embryí, ani u jednodenních kuřat.
- Kuřata infikovaná jako jednodenní vylučovala významně méně oocyst než kuřata infikovaná jako embrya.
- U všech kuřat, která byla infikována jako embrya druhem *C. baileyi* byla specifická DNA této kryptosporidie detekována po vylíhnutí ve všech vyšetřovaných orgánech vyjma mozku. Během šesti dnů po vylíhnutí došlo k vymizení infekce z ledvin, jater, předžaludku a žaludku. Ostatní orgány zůstaly pozitivní až do konce experimentu.
- U kuřat infikovaných *C. baileyi* ve věku jednoho dne po vylíhnutí byla infekce detekována pouze v průdušnici, jícnu, dvanáctníku a jejunoileu.
- U kuřat infikovaných druhem *C. parvum* ve věku jednoho dne po vylíhnutí se infekce rozvinula pouze v tenkém střevě.
- U kuřat infikovaných stejným druhem kryptosporidie ve fázi embrya došlo k rozvoji infekce i v průdušnici, slepém a tlustém střevě a kloace.

- Kuřata infikovaná *C. baileyi* jako embrya uhynula do 16 dnů po vylíhnutí. Všechna ostatní kuřata se z infekce samovyléčila.

Byla zpracována a opakovaně ověřena metodika pro úspěšnou infekci embryí oocystami kryptosporidií.

1. Pro infekci je nutné použít čisté oocysty – purifikované na cesium chloridovém gradientu nebo využít obdobnou metodou.

2. Před infekcí je nutné oocysty dezinfikovat – 5% chlornan sodný po dobu 15 minut při teplotě 4 °C.

3. Ideální den pro inokulaci embrya je 9. den vývoje.

4. Je nezbytné dezinfikovat skořápku vejce a vrták, kterým je do skořápky vyvrtán otvor určený k inokulaci.

5. Prosvícením skořápky vejce najít vzduchovou bublinu, vyvrtat díru o velikosti 2 mm a vstříknout připravenou infekční dávku (50 µl) do alantoisu.

6. Vyvrtaný otvor překrýt rozehřátým, sterilním parafínem.

7. Vejce vložit zpět do líhně k dolíhnutí kuřete.

5. ZÁVĚRY

Detekce kryptosporidií vyskytujících se u ptáků pomocí molekulárních metod.

- Byly detekovány všechny známé ptačí druhy kryptosporidií, kdy nejčastěji detekovaným druhem bylo *C. baileyi* a *C. proventriculi*. Z více jak 20 známých ptačích genotypů bylo detekováno 6 genotypů, z nichž nejvíce zastoupeným genotypem byl *Cryptosporidium* sp. goose genotyp Id a naopak nejméně často byl detekován *Cryptosporidium* sp. avian genotyp I.
- Kromě hostitelsky specifických kryptosporidií byly u ptáků nalezeny i druhy, které jsou typické pro savce (*C. andersoni, C. canis, C. hominis, C. muris* a *C. parvum*).

Biologická a molekulární charakterizace nových druhů a genotypů kryptosporidií

- Byly popsány tři nové ptačí druhy kryptosporidií: *Cryptosporidium avium* sp. n., *Cryptosporidium proventriculi* sp. n. a *Cryptosporidium ornithophilus* sp. n., a dva nové genotypy, z nichž *Cryptosporidium* avian genotyp VI byl v rámci této práci již publikován a genotyp nalezený u kachny divoké bude předmětem dalšího bádání.
- V rámci popisu druhu byla popsána morfologie a morfometrie oocyst, hostitelská a tkáňová specifita a patogenita.
- Kryptosporidiové infekce ptáků nejsou většinou provázeny klinickými příznaky onemocnění.

Kultivace kryptosporidií pomocí kuřecích embryí

- Kuřecí embrya jsou vnímavá k infekci střevními kryptosporidiemi, *C. baileyi* specifickém pro ptáky a *C. parvum*, které je jen omezeně infekční pro ptáky v postnatální fázi vývoje. Byla zavedena metoda kultivace *C. parvum* a *C. baileyi* v kuřecích embryích s mnohonásobně vyšší výtěžností oocyst než u jiných, dosud používaných metod.

6. SEZNAM POUŽITÉ LITERATURY

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7. PŘÍLOHY

7.1 Příloha I

Cryptosporidium galli and novel _Cryptosporidium_ avian genotype VI in North American red-winged blackbirds (_Agelaius phoeniceus_).

Chelladurai J.J., Clark M.E., Kváč M., **Holubová N**., Khan E., Stenger B.L., Giddings C.W., McEvoy J. 2016: Parasitology Research 115: 1901–1906.

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ORIGINAL PAPER

Cryptosporidium galli and novel *Cryptosporidium* avian genotype VI in North American red-winged blackbirds (*Agelaius phoeniceus*)

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Abstract Proventriculus and intestinal samples from 70 North American red-winged blackbirds (*Agelaius phoeniceus*; order Passeriformes) were examined for the presence of *Cryptosporidium* by PCR amplification and sequence analysis of the 18S ribosomal RNA (18S rRNA), actin, and 70-kDa heat shock protein (HSP70) genes. Twelve birds (17.1 %) were positive for the *Cryptosporidium* 18S rRNA gene: six birds were positive at the proventriculus site only and six birds were positive at the proventriculus and intestinal sites. Sequence analysis of the 18S rRNA, actin and HSP70 genes showed the presence of the gastric species *Cryptosporidium galli* in a single proventriculus sample and a closely related genotype, which we have named *Cryptosporidium* avian genotype VI, in all other positive samples. These findings contribute to our understanding of *Cryptosporidium* diversification in passerines, the largest avian order.

Keywords *Cryptosporidium* · Red-winged blackbird · Passerines · *Cryptosporidium galli* · Avian genotype VI · Proventriculus · Intestine

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Introduction

The apicomplexan parasite *Cryptosporidium* infects the gastrointestinal epithelium of all major vertebrate groups (Kváč et al. 2014) and causes the disease cryptosporidiosis, which can be chronic and life threatening (Checkley et al. 2015). Approximately 30 species and 70 genotypes of *Cryptosporidium* form two major clades in nucleotide sequence phylogenies: a smaller, basal clade characterized by specificity for the gastric epithelium and a larger clade comprising members that infect the intestinal epithelium (Xiao et al. 2004).

Cryptosporidium has been identified in 17 of the 26 avian orders, including the largest order, Passeriformes, which contains almost 60 % of the extant avian diversity in 5700 species (Kváč et al. 2014). Passerines are host to three avian-adapted *Cryptosporidium* species, *Cryptosporidium galli*, *Cryptosporidium meleagridis*, and *Cryptosporidium baileyi* and three avian-adapted *Cryptosporidium* genotypes, avian genotypes I, III, and IV (Current et al. 1986; Gomes et al. 2012; Nakamura et al. 2014; Nakamura et al. 2009; Ng et al. 2006; Qi et al. 2011; Ryan 2010; Ryan et al. 2003b; Sevá Ada et al. 2011; Slavin 1955).

Most studies on *Cryptosporidium* in passerines have focused on captive birds, and comparatively little is known about *Cryptosporidium* infecting free-living, wild birds. There has been just one report to date of *Cryptosporidium* in free-living North American passerines: an uncharacterized *Cryptosporidium* species was identified in fledgling cliff swallows (*Petrochelidon pyrrhonota*) with clinical signs of conjunctivitis, rhinitis, and sinusitis (Ley et al. 2012).

Red-winged blackbirds (*Agelaius pheonicius*) are members of the New World passerine family Icteridae, which has 95 species in 23 genera, including oropendolas (*Psarocolius* spp.), caciques (*Cacicus* spp.), orioles (*Icterus*

Table 1 Samples positive for *Cryptosporidium* DNA by PCR analysis of the 18S rRNA, actin, and HSP70 genes

Animal number	Sex	Sample number	Genes			Species/genotype
			18S rRNA	Actin	HSP70	
1	Female	PV-3486	+[a]	−	+	Avian genotype VI
		IN-3487	−[b]	−	−	
2	Female	PV-3492	+	+	+	Avian genotype VI
		IN-3493	−			
3	Male	PV-3543	+	−	−	Avian genotype VI
		IN-3544	−	−	−	
4	Female	PV-3545	+	−	−	Avian genotype VI
		IN-3546	+	−	−	Avian genotype VI
5	Male	PV-3551	+	−	−	Avian genotype VI
		IN-3552	−			
6	Male	PV-3553	+	−	+	Avian genotype VI
		IN-3554	+	−	+	Avian genotype VI
7	Female	PV-3575	+	−	−	Avian genotype VI
		IN-3576	−	−	−	
8	Male	PV-3605	+	+	−	*C. galli*
		IN-3606	−	−	−	
9	Male	PV-3607	+	−	+	Avian genotype VI
		IN-3608	+	−	+	Avian genotype VI
10	Male	PV-3635	+	−	+	Avian genotype VI
		IN-3636	+	−	+	Avian genotype VI
11	Female	PV-18212	+	+	+	Avian genotype VI
		IN-18213	+	−	−	Avian genotype VI
12	Male	PV-18220	+	+	+	Avian genotype VI
		IN-18221	+	+	+	Avian genotype VI

PV proventiculus, *IN* intestine

[a] Positive by PCR analysis

[b] Negative by PCR analysis

spp.), meadowlarks (*Sturnella* spp.), grackles (*Quiscalus* spp., *Hypopyrrhus* spp., *Lampropsar* spp., and *Macroagelius* spp.), and cowbirds (*Molothrus* spp.) (Lowther 1975). They are abundant in North America with a range that extends as far north as Alaska and as far south as Cuba (Yasukawa and Searcy 1995). Higher latitude populations migrate to lower latitudes during winter, where they nest in marshes, wetlands, and hayfields (Ball et al. 1988). To date, the only Icteridae family members identified as hosts of *Cryptosporidium* have been chopi blackbirds (*Gnorimopsar chopi*) from Brazil, which hosted *C. galli* (Nakamura et al. 2014), and a red rumped cacique (*Cacicus haemorrhous*) and crested oropendola (*Psarocolius decumanus*) from the Czech Republic, which hosted *C. baileyi* (Ryan et al. 2003a).

In the present study, *Cryptosporidium* DNA from proventriculus and intestinal contents of red-winged blackbirds caught in the USA was characterized by sequence analysis of the 18S ribosomal RNA (18S rRNA), actin, and heat shock protein 70 (HSP70) genes. These analyses show the presence of *C. galli* and the closely related, novel *Cryptosporidium* avian genotype VI in North American red-winged blackbirds.

Materials and methods

Sample collection and DNA isolation

Seventy red-winged blackbirds, comprising 41 after hatch year males, 26 after hatch year females, and three juveniles of undetermined sex, were captured using live capture (e.g., mist nets, walk-in traps) or lethal methods from areas of Kansas, North Dakota, and Minnesota, USA. Live captured birds were immediately euthanized by over-anesthetizing with halothane. Bird carcasses were dissected and a sample was taken from the proventriculus and intestinal contents. DNA

was isolated from 200 mg of each sample by alkaline digestion and phenol-chloroform extraction and purified using the QIAamp DNA Stool Mini Kit (QIAGEN, Valencia, CA) as described previously (Feltus et al. 2006). The capture, handling, and euthanizing of birds in this study were conducted in accordance with Institutional Animal Care and Use Committee of North Dakota State University Protocol #A13006.

PCR amplification

Nested PCR protocols were used to amplify fragments of the 18S rRNA, actin, and HSP70 genes. A fragment of the *Cryptosporidium* 18S rRNA gene was amplified as described by Xiao et al. (2001), with the exception that 0.5× PCR buffer was used (Promega, Madison, WI). A fragment of the actin gene was amplified as previously described by Sulaiman et al. (2002). The protocol to amplify a fragment of the HSP70 gene was developed as part of this study. Nested PCR primers were designed with specificity for HSP70 sequences that are conserved in *C. galli* [accession no. AY168849], *Cryptosporidium* sp. CzechB1 Eurasian woodcock [accession no. AY273773], *Cryptosporidium muris* [accession no. AF221542], *Cryptosporidium andersoni* [accession no. AY954894], *Cryptosporidium serpentis* [accession no. AF221541], and *Cryptosporidium scrofarum* [accession no. JX424842]. In the primary reaction, a fragment of ~750 bp was amplified using 0.1 μM each of the primers HSPAvAF1

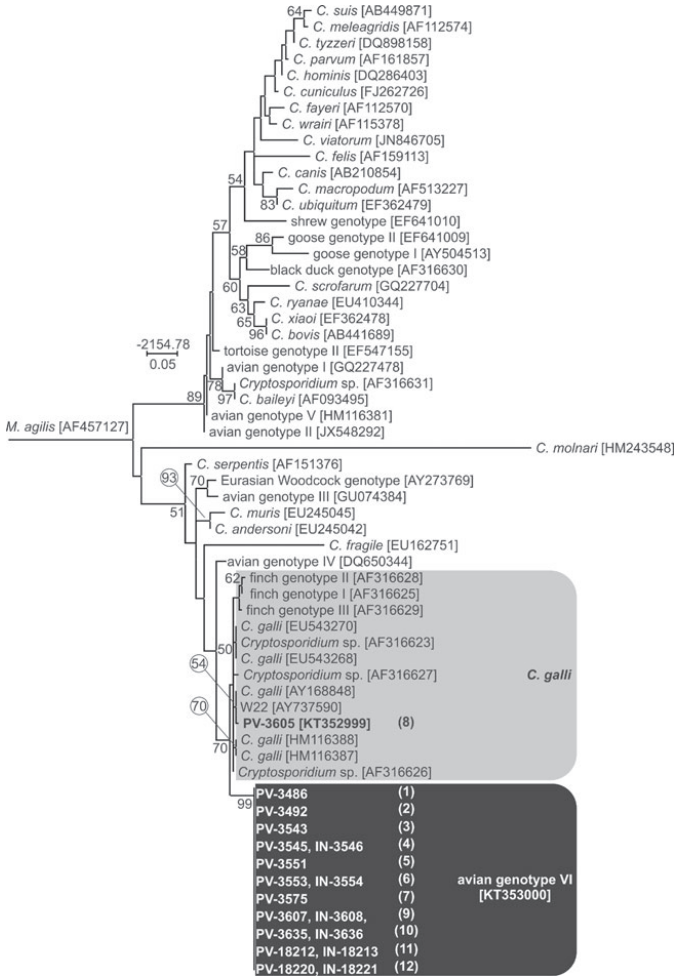

Fig. 1 Maximum likelihood tree of 18S rRNA gene sequences. The evolutionary history was inferred by using the maximum likelihood method based on the general time reversible model (Tavaré 1986). The tree with the highest log likelihood is shown. The percentage of trees in which the associated taxa clustered together is shown above the branches. Initial tree(s) for the heuristic search were obtained automatically by applying neighbor-join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach and then selecting the topology with superior log likelihood value. A discrete gamma distribution was used to model evolutionary rate differences among sites. The rate variation model allowed some sites to be evolutionarily invariable. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The tree was rooted with 18S rRNA from *Monocystis agilis* [accession no. AF457127]. The prefix *PV* indicates a sequence obtained from a proventriculus sample. The prefix *IN* indicates a sequence obtained from an intestinal sample. The animal number from Table 1 is presented in parenthesis after the sample number(s)

(5′-GCT CGT GGT CCT AAA GAT AA) and HSPAvAR1 (5′-ACG GGT TGA ACC ACC TAC TAA T), 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 2.5U Taq DNA polymerase, 1× PCR buffer, and 0.5–2 μL template DNA in a 100-μL reaction. A secondary fragment of ~515 bp was amplified using 0.1 μM each of the primers HSPAvAF2 (5′-ACA GTT CCT GCC TAT TTC) and HSPAvAR2 (5′-GCT AAT GTA CCA CGG AAA TAA TC), 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 2.5U Taq DNA polymerase, 1× PCR buffer, and 2 μL of primary PCR product in a 100-μL secondary reaction. The primary PCR conditions were 35 cycles of 94 °C for 45 s, 52 °C for 45 s, and 72 °C for 1 min. The first cycle was preceded by an initial denaturation at 94 °C for 5 min, and the last cycle was followed by a final extension at 72 °C for 10 min. The secondary reaction used the same conditions as the primary, with the exception that the annealing temperature was 50 °C.

DNA from *Cryptosporidium hominis* was used as a positive control for the 18S rRNA and actin PCR reactions. Water was included instead of DNA template as a negative control in all reactions. Secondary PCR products were separated on an agarose gel and visualized under UV illumination using ethidium bromide staining.

Sequencing and phylogenetic analysis

PCR products were purified (Wizard SV, Promega, Madison, WI) and sequenced in both directions with secondary primers using a BigDye Terminator v3.1 cycle sequencing kit in an ABI Prism 3130 genetic analyzer (Applied Biosystems, Carlsbad, CA). Sequences were assembled using SeqMan (DNAStar, Madison, WI) and aligned using the MAFFT version 7 online server with automatic selection of alignment strategy (http://mafft.cbrc.jp/alignment/server/) (Katoh and Standley 2013). Alignments were manually edited and phylogenetic analyses were performed using MEGA 6.0 (Tamura et al. 2013). The evolutionary history of aligned sequences

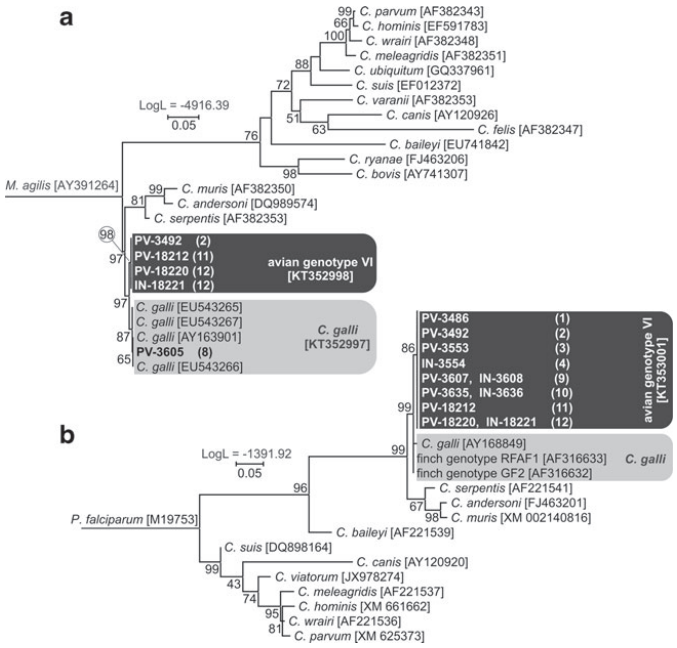

Fig. 2 Maximum likelihood tree of actin (**a**) and HSP70 (**b**) gene sequences. The evolutionary history was inferred by using the maximum likelihood method based on the general time reversible model (Tavaré 1986) for the actin tree or the Tamura 3-parameter model (Tamura 1992) for the HSP70 tree. The tree with the highest log likelihood is shown. The percentage of trees in which the associated taxa clustered together is shown above the branches. Initial tree(s) for the heuristic search were obtained automatically by applying neighbor-join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach and then selecting the topology with superior log likelihood value. A discrete gamma distribution was used to model evolutionary rate differences among sites in actin sequences. The rate variation model allowed some sites in actin and HSP70 sequences to be evolutionarily invariable. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The actin tree was rooted with an actin sequence from *Monocystis agilis* [accession no. AY391264]. The HSP70 tree was rooted with a HSP70 sequence from *Plasmodium falciparum* [accession no. M19753]. The prefix *PV* indicates a sequence obtained from a proventriculus sample. The prefix *IN* indicates a sequence obtained from an intestinal sample. The animal number from Table 1 is presented in parenthesis after the sample number(s)

was inferred using the maximum likelihood (ML) method (Saitou and Nei 1987), with the substitution model that best fit the alignment selected using the Bayesian information criterion. The general time reversible model (Tavaré 1986) with a gamma rate distribution and invariant sites was selected for 18S rRNA and actin alignments. The Tamura 3-parameter model (Tamura 1992) with invariant sites was selected for the HSP70 alignment.

Sequences from this study have been deposited in GenBank under the accession numbers KT352997–KT353001.

Results

Prevalence of *Cryptosporidium* in red-winged blackbirds

Twelve out of 70 birds (17.1 %), seven males and five females, were positive for the *Cryptosporidium* 18S rRNA gene (Table 1). Six birds were positive only at the proventriculus site and six birds were positive at both the proventriculus and intestinal sites.

Phylogenetic analysis of *Cryptosporidium* isolates from red-winged blackbirds

A ML tree constructed from 18S rRNA gene sequences in this study and representative sequences in GenBank showed the presence of two closely related genotypes in the gastric *Cryptosporidium* clade (Fig. 1). PV-3605 clustered with *C. galli*, sharing 99.9 % sequence identity with a *C. galli* isolate from storm water in New York, USA [accession no. AY737590]. Sequences from the remaining 17 samples shared 100 % identity with each other, 98.5 % identity with PV-3605, and between 97.7 and 98.3 % identity with *C. galli* sequences published in GenBank. We have named this novel genotype *Cryptosporidium* avian genotype VI.

A fragment of the actin gene was amplified and sequenced from five of the 18 samples that were positive for the 18S rRNA gene (Fig. 2a). PV-3605 shared 100 % sequence identity with *C. galli* from a western capercaillie [accession no. AY163901], chestnut-bellied seed finch [accession no. EU543267], Atlantic canary [accession no. EU543266], and cockatiel [accession no. EU543265]. Actin sequences from *Cryptosporidium* avian genotype VI (PV-3492, PV-18212, PV-18220, and IN-18221) shared 100 % identity with each other and 99.3 % identity with PV-3605 and published actin sequences from *C. galli*.

A fragment of the HSP70 gene was amplified and sequenced from 11 of the 18 samples that were positive for the 18S rRNA gene (Fig. 2b). A HSP70 sequence could not be obtained from the *C. galli* isolate PV-3605. Sequences of HSP70 from *Cryptosporidium* avian genotype VI shared 100 % identity with each other and 98.6 % identity with a published *C. galli* HSP70 sequence [accession no. AY168849].

Discussion

We report on the genotyping of *Cryptosporidium* isolates from red-winged blackbirds in the USA. Data show that free-living North American red-winged blackbirds host the gastric species *C. galli*, and a closely related genotype within the gastric clade, which we have named *Cryptosporidium* avian genotype VI.

Consistent with their phylogenetic positions, *C. galli* and avian genotype VI were found in the proventriculus of all positive red-winged blackbirds. The finding that half of the positive birds also had a positive intestinal sample is not surprising, as oocysts of gastric species pass through the intestine and are shed in the feces. Although we did not perform histopathology to confirm the gastric location, previous work has shown that *C. galli* exclusively infects the proventricular epithelium (Blagburn et al. 1990; Morgan et al. 2001), similar to the Eurasian woodcock genotype (Ryan et al. 2003a) and avian genotype III (Makino et al. 2010). Each of these species/genotypes has been shown to cause clinical disease and mortality in birds (Blagburn et al. 1990; Makino et al. 2010; Morgan et al. 2001; Ryan et al. 2003a). Further studies are required to determine if *Cryptosporidium* avian genotype VI is pathogenic for red-winged blackbirds.

The 18S rRNA gene in *C. galli* exhibits significant sequence heterogeneity (Morgan et al. 2001). In one study, three different 18S rRNA sequences from a single bird diverged by 0.6 ± 0.3 % (Morgan et al. 2001). These sequences were initially named finch genotypes I, II, and III, but were subsequently classified as heterogeneous sequences of *C. galli* (Ryan et al. 2003b). Among the possible explanations for intraspecific 18S rRNA sequence heterogeneity, the occurrence of paralogous gene copies (Le Blancq et al. 1997; Stenger et al. 2015) is most problematic for the inference of evolutionary relationships because paralogs are not related by descent (Koonin 2005). The mean evolutionary divergence of 18S rRNA sequences from avian genotype VI and *C. galli* (2.0 ± 0.5 %) was greater than that of heterogeneous sequences from *C. galli* (0.7 ± 0.2 %; calculated using Mega 6.0 from sequences reported in Fig. 1), supporting our decision to categorize avian genotype VI separately from *C. galli*. The finding that of actin and HSP70 sequences also diverged further supported this decision.

The present study has contributed to the understanding of *Cryptosporidium* diversity in passerine hosts. Further sampling of passerines in their natural habitat will help to determine how factors such as host speciation and geographic

isolation have influenced *Cryptosporidium* diversification in birds.

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7.2 Příloha II

***Cryptosporidium avium* n. sp. (Apicomplexa: Cryptosporidiidae) in birds.**

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ORIGINAL PAPER

Cryptosporidium avium n. sp. (Apicomplexa: Cryptosporidiidae) in birds

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Abstract The morphological, biological, and molecular characteristics of *Cryptosporidium* avian genotype V are described, and the species name *Cryptosporidium avium* is proposed to reflect its specificity for birds under natural and experimental conditions. Oocysts of *C. avium* measured 5.30–6.90 μm (mean = 6.26 μm) × 4.30–5.50 μm (mean = 4.86 μm) with a length to width ratio of 1.29 (1.14–1.47). Oocysts of *C. avium* obtained from four naturally infected red-crowned parakeets (*Cyanoramphus novaezealandiae*) were infectious for 6-month-old budgerigars (*Melopsittacus undulatus*) and hens (*Gallus gallus f. domestica*). The prepatent periods in both susceptible bird species was 11 days postinfection (DPI). The infection intensity of *C. avium* in budgerigars and hens was low, with a maximum intensity of 5000 oocysts per gram of feces. Oocysts of *C. avium* were microscopically detected at only 12–16 DPI in hens and 12 DPI in budgerigars, while PCR analyses revealed the presence of specific DNA in fecal samples from 11 to 30 DPI (the conclusion of the experiment). *Cryptosporidium avium* was not infectious for 8-week-old SCID and BALB/c mice (*Mus musculus*). Naturally or experimentally infected birds showed no clinical signs of cryptosporidiosis, and no pathology was detected. Developmental stages of *C. avium* were detected in the ileum and cecum using scanning electron microscopy. Phylogenetic analyses based on small subunit rRNA, actin, and heat shock protein 70 gene sequences revealed that *C. avium* is genetically distinct from previously described *Cryptosporidium* species.

Keywords *Cryptosporidium avium* · Morphology · Molecular analyses · Transmission studies · *Cryptosporidium* avian genotype V

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Introduction

Cryptosporidium parasites belong to the phylum Apicomplexa and infect the gastrointestinal tract of a broad range of vertebrate species (Fayer 2010), causing the diarrheal disease cryptosporidiosis. Currently, around 30 species of *Cryptosporidium* infecting fish, amphibians, reptiles, birds, and mammals are considered to be valid (Kváč et al. 2014a; Liu et al. 2013; Qi et al. 2011). Of these, only three have specificity for birds: *Cryptosporidium meleagridis*, *Cryptosporidium baileyi*, and *Cryptosporidium galli* (Current et al. 1986; Ryan et al. 2003b; Slavin 1955). In addition, 11 *Cryptosporidium* genotypes have been described in more than 30 bird species worldwide, including avian I–V, goose genotypes I–IV, duck genotype, and Euroasian Woodcock genotype (Ryan 2010). Of these, only *C. meleagridis* is known to also infect humans (Alves et al. 2003; Cama et al. 2003; McLauchlin et al. 2000; Xiao and Ryan 2004). Although mammal-specific *Cryptosporidium* species and genotypes are rarely detected in birds, *Cryptosporidium hominis*, *C. hominis*-like, *Cryptosporidium parvum*, and muskrat genotype I have been reported in fecal samples from Canada geese (*Branta canadensis*) (Graczyk et al. 1998; Jellison et al. 2004, 2009; Zhou et al. 2004).

Natural cryptosporidiosis of birds caused by *C. meleagridis* and *C. galli* affects the gastrointestinal tract and manifests in different degrees of enteritis (Gharagozlou et al. 2006; Ryan et al. 2003b), whereas *C. baileyi* infects many sites, including conjunctiva, nasopharynx, trachea, bronchi, air sac, gut, bursa of Fabricius, kidneys, and urinary tract, and manifests in three clinical forms: respiratory disease, enteritis, and renal disease (Lindsay and Blagburn 1990). Usually, only one form of the disease is present in an outbreak (Lindsay and Blagburn 1990). Also, *Cryptosporidium* avian genotype III was reported as a possible cause of chronic vomiting in peach-faced lovebirds (*Agapornis roseicollis*) (Makino et al. 2010). Pathogenicity has not been described for other bird-derived *Cryptosporidium* genotypes (Ng et al. 2006).

The redescription of *Cryptosporidium* genotypes as new species requires morphometric studies of oocysts, genetic characterizations, and demonstration of host specificity (natural and, where possible, experimental) (Xiao et al. 2004). These data have thus far been lacking for *Cryptosporidium* genotypes from birds (Ng et al. 2006). The present study aimed to address this deficiency for *Cryptosporidium* avian genotype V, a genotype first reported in cockatiels (*Nymphicus hollandicus*) in Japan (Abe and Makino 2010) and subsequently in many other bird hosts (Table 1). Based on the collective data from this and other studies, we conclude that *Cryptosporidium* avian V is genetically and biologically distinct from recognized *Cryptosporidium* species, and we propose that it be named *Cryptosporidium avium*.

Materials and methods

Source of oocysts for studies

Oocysts of *C. avium* were originally isolated from fecal samples of four naturally infected adult red-crowned parakeets (*Cyanoramphus novaezealandiae*), which were caged by a private owner in České Budějovice (Czech Republic). *Cryptosporidium avium* oocysts from these red-crowned parakeets were pooled and used to infect a single 6-month-old hen (hen 1; *Gallus gallus f. domestica*). Oocysts from hen 1 were used to infect other animals (see "Transmission studies" section).

Parasitological examination and oocyst preparation

Animal feces were screened for *Cryptosporidium* oocysts using fecal smears stained with aniline-carbol-methyl violet (ACMV) (Miláček and Vítovec 1985). Fecal specimens were collected daily and stored in a 2.5 % potassium dichromate solution at 4–8 °C.

Cryptosporidium oocysts originated from red-crowned parakeets and from hen 1 were purified using cesium chloride gradient centrifugation for morphometry analyses and transmission studies (Kilani and Sekla 1987). The viability of oocysts was examined using propidium iodide (PI) staining by a modified assay of Sauch et al. (1991). Briefly, examined oocysts were washed in distilled water (DW; 10,000 oocysts in 100 μl) and mixed with 1 μl of PI (1 % solution, Sigma). After 30 min of incubation at room temperature in the dark, the oocysts were washed twice with DW. Oocyst viability was examined using fluorescence microscopy (filter 420 nm, Olympus IX70). Oocysts with red fluorescence were considered to be dead, and those without fluorescence were considered viable.

Oocyst morphology

Cryptosporidium avium oocysts for morphology and morphometry analyses were examined using differential interference contrast (DIC) microscopy, brightfield microscopy following ACMV staining, and fluorescence microscopy

Table 1 Occurrence of *Cryptosporidium avium* n. sp. (previously known as avian genotype V) demonstrated on the basis of partial sequences of SSU, actin, and HSP70 in various bird hosts in the world

Host (scientific name)	Location	Genes (GenBank accession number)	References
Cockatiel (*Nymphicus holandicus*)	Japan	SSU (AB471646); actin (AB471660); HSP70 (AB471665)	Abe and Makino (2010)
	China	SSU (HM116381)	Qi et al. (2011)
	China	SSU (JQ246415); actin (JQ320301)	unpublished
	China	SSU (KM267556)[a]	Zhang et al. (2015)
Chicken (*Gallus gallus*)	China	SSU (JX548299)	Wang et al. (2014)
Blue-fronted Amazon (*Amazona aestiva*)	Brazil	SSU (KJ487974)	Nakamura et al. (2014)
Major Mitchell's Cockatoo (*Lophochroa leadbeateri*)	USA	SSU (KP342400)	Curtiss et al. (2015)
Budgerigar (*Melopsittacus undulates*)	China	SSU (KM267556)[a]	Zhang et al. (2015)

[a] Identical GenBank accession number for sequence acquired from two different hosts cockatiel and budgerigar

SSU small ribosomal subunit rRNA, *HSP70* 70-kDa heat shock protein

following labeling with genus-specific FITC-conjugated antibodies (*Cryptosporidium* IF Test, Crypto Cel, Medac) (Olympus IX70 microscope, filter 520 nm). Morphology and morphometry were determined using digital analysis of images (M.I.C. Quick Photo Pro v.3.0 software; Optical Service, Czech Republic) collected using a Camedia C-5060 Wide Zoom 5.1 megapixel digital camera (Optical Service). A 20-μl aliquot containing ~10,000 purified oocysts was examined for each measurement. Length and width of oocysts ($n = 100$) were measured under DIC at ×1000 magnification, and these were used to calculate the length-to-width ratio of each oocyst. As a control, the morphometry of *C. baileyi* ($n = 100$) from a naturally infected adult common peafowl (*Pavo cristatus*) were measured by the same person using the same microscope. Photomicrographs of *C. avium* (avian genotype V) oocysts observed by DIC, ACMV, and IFA were deposited as a phototype at the Institute of Parasitology, Biology Centre of the Czech Academy of Sciences, Czech Republic.

DNA extraction and molecular analyses

Total DNA was extracted from 200 mg of feces, 10,000 purified oocysts, or 200 mg of tissue by bead disruption for 60 s at 5.5 m/s using 0.5-mm glass beads in a FastPrep®-24 Instrument (MP Biomedicals, CA, USA). DNA was isolated and purified using a commercially available kit in accordance with the manufacturer's instructions (QIAamp® DNA Stool Mini Kit or DNeasy® Blood & Tissue Kit, Qiagen, Hilden, Germany). Purified DNA was stored at −20 °C prior to being used for PCR. A nested PCR approach was used to amplify a region of the small subunit (SSU) (~830 bp; Jiang et al. 2005; Xiao et al. 1999), actin (~1066 bp; Sulaiman et al. 2002) and HSP70 genes (~1950 bp; Sulaiman et al. 2000). Both primary and secondary PCR reactions were carried out in a volume of 50 μl; the primary reaction contained 2 μl of genomic DNA (or water as a negative control) and the secondary reaction contained 2 μl of the primary reaction as template. DNA of *C. parvum* and *C. baileyi* was used as positive control. Secondary PCR products were detected by agarose gel (2 %) electrophoresis, visualized by ethidium bromide staining, and extracted using QIAquick® Gel Extraction Kit (Qiagen). Purified secondary products were sequenced in both directions with an ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) using the secondary PCR primers and the BigDye1 Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) in 10-μl reactions.

Phylogenetic analyses

The nucleotide sequences of each gene obtained in this study were edited using the ChromasPro 1.7.5 software (Technelysium Pty Ltd.) and aligned with each other and with reference sequences from GenBank using MAFFT version 7 online server with automatic selection of alignment mode (http://mafft.cbrc.jp/alignment/software/). Phylogenetic analyses were performed, and best DNA/protein phylogeny models were selected using the MEGA6 software (Guindon and Gascuel 2003; Tamura et al. 2011). Phylogenetic trees were inferred by the maximum likelihood (ML) method, with the substitution model that best fits the alignment selected using the Bayesian information criterion. The Tamura 3-parameter model (Tamura 1992) was selected for SSU and HSP70 alignments, and the general time reversible model (Tavaré 1986) was selected for actin alignment. Bootstrap support for branching was based on 1000 replications. Phylograms were drawn using the MEGA6 and were manually adjusted using CorelDraw X7. Sequences of SSU, actin, and HSP70 derived in this study have been deposited in GenBank under accession numbers KU058875–KU058886.

Transmission studies

Animals

Three 8-week-old severe combined immunodeficiency (SCID) mice (strain C.B-17), three 8-week-old BALB/c mice (Charles River, Germany), three 6-month-old hens (hen 2–4; *Gallus gallus f. domestica*), and three 6-month-old budgerigars (bud 1–3; *Melopsittacus undulatus*) were used for experimental infection studies. In addition, three animals from each host species/strain were used as negative control.

Experimental design

To prevent environmental contamination with oocysts, laboratory rodents were housed in plastic cages and supplied with a sterilized diet (TOP-VELAZ, Prague, Czech Republic) and sterilized water ad libitum. Hens and budgerigars were kept in species-appropriate birdcages with sterilized wood-chip bedding and without bedding, respectively, and were supplied with sterilized food and water ad libitum. Each animal was inoculated orally by stomach tube with 100,000 purified viable oocysts suspended in 200 μl of distilled water. Animals serving as negative controls were inoculated orally by stomach tube with 200 μl of distilled water. Fecal samples from all animals were screened daily for the presence of *Cryptosporidium* oocyst using ACMV staining, and the presence of *Cryptosporidium*-specific DNA was confirmed using nested PCR targeting the SSU gene. All experiments were terminated 30 days postinfection (DPI). Infection intensity was reported as the number of oocysts per gram (OPG) of feces as previously described by Kváč et al. (2007). In addition, fecal consistency and color and general health status were examined daily. One *C. avium*-positive animal from each

host group was euthanized 20 DPI. Tissue specimens were processed for PCR detection, histology, and electron microscopy.

Histopathological examinations

The complete examination of all gastrointestinal organs was conducted at necropsy. Tissue specimens from the stomach, small intestine, and large intestine (the entire tract was divided into 1-cm sections) were sampled and processed for histology according to Kváč and Vítovec (2003) and for PCR analyses (see "Oocyst morphology" section). Histology sections were stained with hematoxylin and eosin (HE), Wolbach's modified Giemsa stain, and genus-specific FITC-conjugated monoclonal antibodies targeting *Cryptosporidium* oocyst wall antigens (*Cryptosporidium* IF Test, Crypto Cel, Medac).

Scanning electron microscopy

Samples of intestinal tissue originating from a host confirmed to be infected with *C. avium* were fixed in freshly prepared 3 % glutaraldehyde (*v*/*v*) in cacodylate buffer (0.1 M, pH 7.4) at 4 °C and further processed for SEM as described in Valigurová et al. (2008). All samples were examined by JEOL JSM-7401F.

Animal care

Animal caretakers wore new disposable coveralls, shoe covers, and gloves every time they entered the experimental room. All wood-chip bedding, feces, and disposable protective clothing were sealed in plastic bags, removed from the experimental room, and incinerated. All housing, feeding, and experimental procedures were conducted under protocols approved by the Institute of Parasitology, Biology Centre, and Central Commission for Animal Welfare, Czech Republic (protocol nos. 071/2010 and 114/2013).

Results

In the present study, *C. avium* was detected in naturally infected red-crowned parakeets (*C. novaezealandiae*) ($n=4$), which continuously shed oocysts for more than 5 months.

Oocyst morphology

Oocysts of *C. avium* originated from naturally infected red-crowned parakeets were morphometrically identical to those recovered from experimentally infected hen no. 1, measuring 5.30–6.90 μm (mean = 6.26 μm) × 4.30–5.50 μm (mean = 4.86 μm) with a length-to-width ratio of 1.29 (1.14–1.47) ($n=100$; Fig. 1a), and they were smaller than oocysts of *C. baileyi*, measuring 5.90–7.60 μm (mean = 6.90 μm) × 4.30–6.60 μm (mean = 5.50 μm) with a length-to-width ratio of 1.25 (1.06–1.43) ($n=100$; Fig. 1). Oocysts in fecal smears showed typical *Cryptosporidium* ACMV staining characteristics (Fig. 1b). Fixed *C. avium* oocysts labeled with FITC-conjugated anti-*Cryptosporidium* oocyst wall antibody and examined by fluorescence microscopy had typical apple green, halo-like fluorescence (Fig. 1c).

Molecular characterization

At the SSU locus, all isolates of *C. avium* (from naturally infected red-crowned parakeets and experimentally infected hens and budgerigars) shared 100 % identity with each other and with *Cryptosporidium* avian genotype V obtained from cockatiels in Japan (AB471646, AB471647) and China (HM116381). At the actin locus, *C. avium* isolates from all experimentally susceptible hosts shared 100 % identity with each other and with the GenBank sequences of *Cryptosporidium* avian genotype V obtained from cockatiels in Japan (AB471660, AB471661) and China (JQ320301). At HSP70 locus, all sequences of *C. avium* isolates were identical to sequences obtained from a cockatiel (AB471665) and from a rosy-faced lovebird (*A. roseicollis*; AB538401) in Japan. Maximum likelihood trees inferred from sequences of individual genes (data no shown) and concatenated SSU, actin, and HSP70 sequences (Fig. 2) showed that *C. avium* is most closely related to *Cryptosporidium* avian genotype II and also clusters with *Cryptosporidium* avian genotype I and *C. baileyi*.

Experimental transmission studies

Oocyst used for experimental infections had >90 % viability, determined by PI staining. Experimentally inoculated SCID and BALB/c mice did not produce detectable *C. avium* oocysts by microscopy or specific DNA by PCR in fecal samples within 30 DPI. No clinical signs of cryptosporidiosis were detected in any laboratory rodent. Histological and molecular examination of gastrointestinal tract tissue from these rodents did not reveal the presence of *Cryptosporidium* developmental stages or *Cryptosporidium*-specific DNA.

Cryptosporidium avium was fully infectious for all hens and budgerigars. Oocysts were microscopically detected by 12 DPI in both hens and budgerigars. Oocysts of *C. avium* were microscopically detected 12–16 DPI in hens and 12 DPI in budgerigars. The infection intensity of *C. avium* in hens and budgerigars was generally low—hens shed oocysts in range 2000 to 5000 OPG, while budgerigars did not shed more than 2000 OPG. Specific DNA of *C. avium* was detected in feces of both hens and budgerigars from 11 DPI and then intermittently until the end of the experiment. Infected birds showed no symptoms of the disease, and hens and budgerigars necropsied at 20 or 30 DPI showed no macroscopic signs of

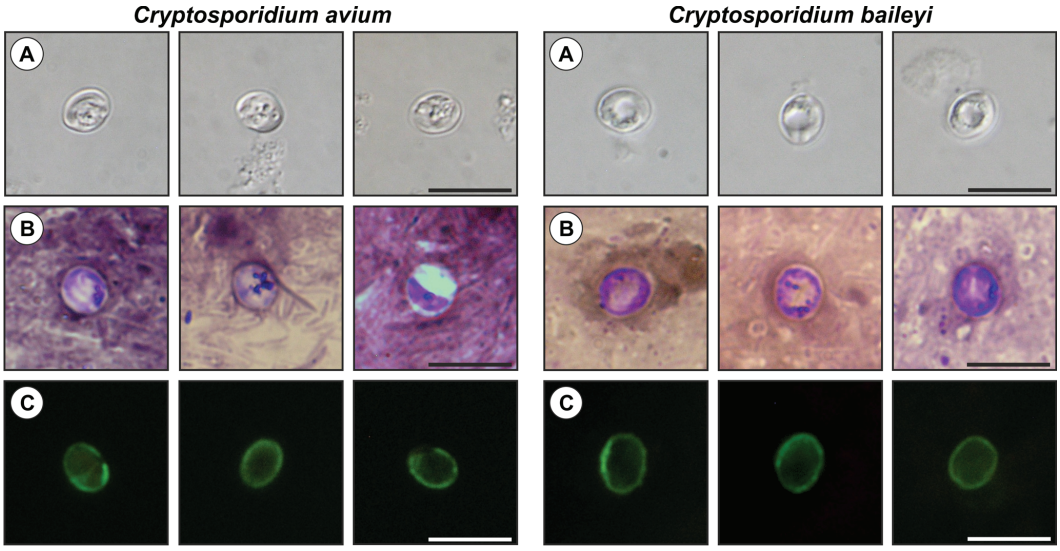

Fig. 1 *Cryptosporidium avium* and *Cryptosporidium baileyi* oocysts visualized in various preparations: **a** differential interference contrast microscopy, **b** aniline–carbol–methyl violet staining, and **c** labeled with anti-*Cryptosporidium* FITC-conjugated antibody. Bar = 10 μm

cryptosporidiosis. No developmental stages of *C. avium* were histologically observed in either hens or budgerigars. However, scanning electron microscopy revealed the presence of developmental stages of *C. avium* attached to the microvilli in the ileum and cecum of hens (Fig. 3) and budgerigars. No pathology-associated changes were observed.

Taxonomic summary

Cryptosporidium avium

Diagnosis: Oocysts are shed fully sporulated. Sporulated oocysts ($n = 100$) measure 5.30–6.90 μm (mean = 6.26 μm) × 4.30–5.50 μm (mean = 4.86 μm) with a length-to-width ratio of 1.29 (1.14–1.47). Endogenous stages are unknown.

Type host: red-crowned parakeet (*C. novaezealandiae*)

Other natural hosts: rosy-faced lovebird (*A. roseicollis*), chicken (*Gallus gallus*), blue-fronted Amazon (*Amazona aestiva*), major Mitchell's cockatoo (*Lophochroa leadbeateri*), cockatiel (*N. hollandicus*), budgerigar (*M. undulatus*) (Table 1)

Experimental hosts: hen (*Gallus gallus domesticus*), budgerigar (*M. undulatus*)

Prepatent period: 11 DPI

Patent period: at least 30 DPI

Type locality: České Budějovice, Czech Republic

Other localities: Brazil, China, Japan, USA

Site of infection: ileum, cecum (this study), kidney, ureter, and cloaca (Curtiss et al. 2015)

Material deposited: A phototype, description of oocysts, and DNA are deposited at the Institute of Parasitology, Biology Centre of the Academy of Sciences of the Czech Republic.

DNA sequences: Partial sequences of SSU, actin, and HSP70 genes were submitted to GenBank under the accession numbers KU058875–KU058886.

Etymology: The species name *avium* is derived from the Latin noun "avis" (meaning a bird) according to ICZN Article 11.9.1–3 as a plural in the genitive case, as it appears to be adapted to birds.

Morphological, genetic, and biological data support the establishment of *Cryptosporidium* avian genotype V as a new species. According to ICZN and criteria for naming species, we propose the name *Cryptosporidium avium*.

Discussion

Avian-adapted *Cryptosporidium* species and genotypes appear to infect a broad range of bird species (Ryan 2010). This is supported by our finding that *C. avium* could be transmitted from parrots, which are in the order Psittaciformes, to hens, which are in the order Galliformes. It is therefore unsurprising that the host range of *C. avium* overlaps that of other avian-adapted *Cryptosporidium,* including the closely related avian genotype II (Abe and Makino 2010). In contrast to *C. meleagridis*, which has been reported in calves, pigs, rabbits, rats, mice, and humans (Akiyoshi et al. 2003; Cama et al.

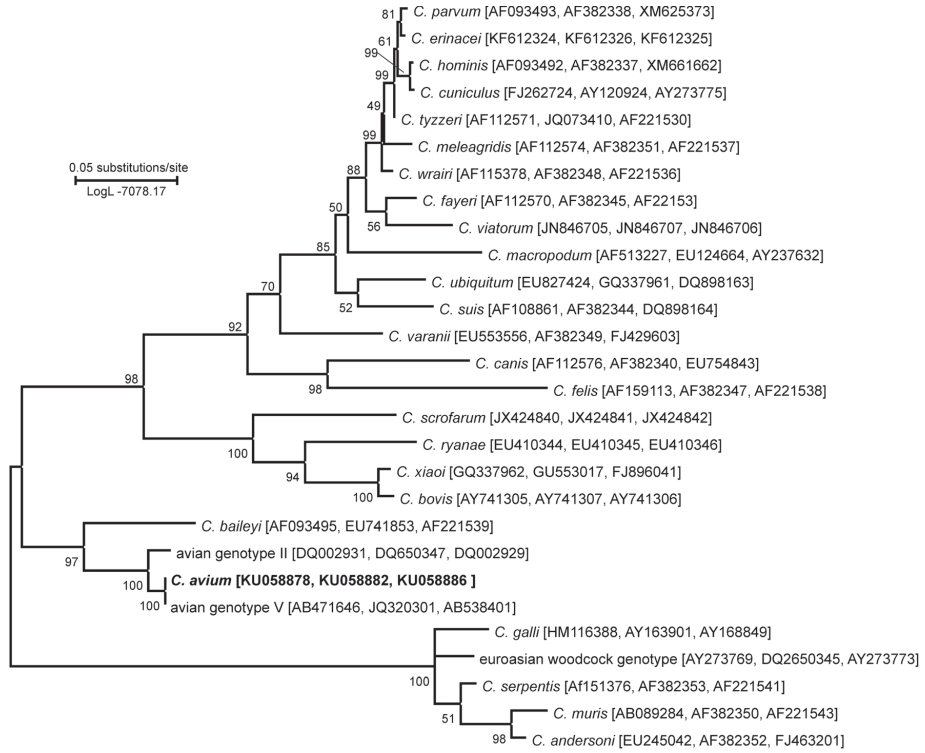

Fig. 2 Phylogenetic relationships between *Cryptosporidium avium* and selected *Cryptosporidium* spp. as inferred by a maximum likelihood (ML) analysis of concatenated sequences constructed from partial DNA sequences of SSU, actin, and HSP70 loci (1234 base positions in the final dataset; model Tamura 3-parameter G + I). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates). *Numbers* at the nodes represent bootstrap values for the nodes gaining more than 50 % support. *Scale bar* included in tree

2003; Darabus and Olariu 2003; Elwin et al. 2012; Huang et al. 2003; O'Donoghue 1995; Xiao and Ryan 2004), there is no evidence that *C. avium* infects non-avian hosts (present study; Kváč et al. 2014b).

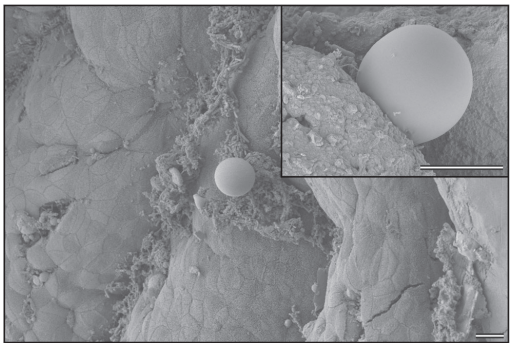

Fig. 3 Scanning electron photomicrograph. Epithelium of ileum of a hen, sacrificed 20 DPI, showing attached *Cryptosporidium avium*. Detail of the attached *C. avium* developmental stage is provided in the *upper right corner*. Bar = 10 μm

Most birds infected with *C. avium*, including experimentally infected hens and budgerigars, showed no clinical signs of cryptosporidiosis (present study; Ng et al. 2006); however, a 7-year-old Major Mitchell's cockatoo (*L. leadbeateri*) showed signs of lethargy, anorexia, and cloacal prolapse (Curtiss et al. 2015). *Cryptosporidium avium* was detected in the kidneys, ureter, and cloaca of the Major Mitchell's cockatoo, and developmental stages were found in the ileum and cecum in the present study. This broad tissue tropism is similar to the genetically related species and genotype, *C. baileyi* and avian genotype II (Nakamura and Meireles 2015).

Until now, the course of *Cryptosporidium* infection in birds has been described only for *C. meleagridis*, *C. baileyi*, and *C. galli* (Current et al. 1986; Ryan et al. 2003b; Slavin 1955). We have shown that the prepatent period of *C. avium* (12 days) is significantly longer than that of *C. meleagridis* and *C. baileyi* (4–8 days; Hornok et al. 1998; Lindsay et al. 1988; Rhee et al. 1991; Tůmová et al. 2002) and shorter than

that of *C. galli* (25 days, Pavlásek 2001). Differences in the prepatent period of *Cryptosporidium* species are not unusual, even for phylogenetically closely related species infecting the same host. For example, *Cryptosporidium bovis* and *Cryptosporidium ryanae* have a similar host range (cattle) and share 98 % sequence identity at the SSU locus, but *C. ryanae* has a shorter prepatent period (11 days) than *C. bovis* (16 days) (Fayer et al. 2005, 2008).

Although infected birds shed low numbers of *C. avium* oocysts, shedding continued for the duration of experimental infections (30 DPI), and naturally infected red-crowned parakeets continued to shed oocysts for at least 5 months. A several month-long natural infection was previously observed in various passerines naturally infected with the gastric species *C. galli*. The reported duration of *C. baileyi* and *C. meleagridis* infections ranges from 4 to 151 and 4 to 21 days, respectively, depending on species and age of the host (Bermudez et al. 1988; Sreter et al. 1995; Tůmová et al. 2002; Woodmansee et al. 1988).

Cryptosporidium avium oocysts from this study (5.30–6.90 × 4.30–5.50 μm) are morphometrically indistinguishable from those of *Cryptosporidium* avian genotype V (5.0–6.6 × 4.1–5.2 μm, Qi et al. 2011), similar to those of *Cryptosporidium* avian genotype II (6.0–6.5 × 4.8–6.6 μm, Meireles et al. 2006; Ng et al. 2006; Qi et al. 2011) and *C. baileyi* (6.3 × 4.6 μm, Current et al. 1986), larger than those of *C. meleagridis* (5.0 × 4.3 μm, Slavin 1955), and smaller than those of *C. galli* (8.0–8.5 × 6.2–6.4 μm, Ryan et al. 2003b), *Cryptosporidium* avian III (7.5 × 6.3 μm, Meireles et al. 2006; Ng et al. 2006), and Euroasian woodcock genotype (8.5 × 6.4 μm, Ryan et al. 2003a).

Phylogenetic analyses based on SSU, actin, and HSP70 gene sequences showed that *C. avium* is genetically distinct from known species and is most closely related to *C. bailey* and *Cryptosporidium* avian genotypes I and II.

At the SSU locus, *C. avium* exhibits 1.70 and 0.28 % genetic distance from avian genotypes I and II, respectively, and 2.27 % genetic distance from *C. baileyi*. At the actin locus, the genetic distance from avian genotypes I and II is 10.8 and 1.86 %, respectively, and the genetic distance from *C. baileyi* is 11.04 %. At the HSP70 locus, *C. avium* exhibits 4.49 and 12.92 % genetic distance from avian genotype II and *C. baileyi*, respectively. These differences are comparable to genetic distances of currently accepted species. For example, at the SSU, actin, and HSP70 loci, the respective genetic distances between *C. parvum* and *C. erinacei* is 0.42, 0.41, and 0.72 %; *C. hominis* and *C. cuniculus* is 1.11, 0.37, and 1.65 %; and *C. muris* and *C. andersoni* is 0.70, 3.54, and 2.21 % at SSU, actin, and HSP70 loci, respectively.

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7.3 Příloha III

***Cryptosporidium meleagridis* and *C. baileyi* (Apicomplexa) in domestic and wild birds in Algeria.**

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Cryptosporidium meleagridis and *C. baileyi* (Apicomplexa) in domestic and wild birds in Algeria

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Abstract: A total of 345 faecal samples were collected from domestic, captive and wild birds in rural areas, urban areas and a Zoo in Algeria. Samples were screened for the presence of parasites belonging to the genus *Cryptosporidium* Tyzzer, 1910 by microscopy and PCR analysis of the small-subunit rRNA (SSU), actin and 60-kDa glycoprotein (gp60) genes. *Cryptosporidium* spp. were detected in 31 samples. Sequence analysis of SSU and actin genes revealed the presence of *C. baileyi* Current, Upton et Haynes, 1986 in domestic chicken broilers (n = 12), captive ostriches (n = 4) and a wild mallard (n = 1), and *C. meleagridis* Slavin, 1955 in a graylag goose (n = 1), chickens (n = 11) and turkeys (n = 2). Twenty-three chicken and two turkey broilers from five farms were positive for cryptosporidia, with an overall prevalence of 2% and 6%, respectively. Both *C. meleagridis* and *C. baileyi* were detected in farmed chicken broilers, with a prevalence ranging from 9% to 69%. Farmed turkeys broilers were positive only for *C. meleagridis*, with a 13% prevalence at the animal level. Subtyping of *C. meleagridis* isolates at the gp60 locus showed the presence of subtype IIIgA22G3R1 in graylag goose and chicken broilers and IIIgA23G2R1 in chicken and turkey broilers. Infection with cryptosporidia was not associated with any clinical diseases. The results of the present study, which provides the first data on the prevalence of *Cryptosporidium* spp. in wild birds in Africa, demonstrate the presence of human pathogenic *C. meleagridis* in both domestic and wild birds in Algeria.

Keywords: avian cryptosporidia, PCR, epidemiology, Northern Africa

Birds are important to public and animal health because they carry various pathogens, including the zoonotic parasites of the genus *Cryptosporidium* Tyzzer, 1910 (see Reed et al. 2003, Graczyk et al. 2008). The genus *Cryptosporidium* comprises species of apicomplexan parasites that infect epithelial cells in the microvillus border of the gastrointestinal tract of all vertebrate classes (O'Donoghue 1995). Species of genus *Cryptosporidium* additionally infect the bursa of Fabricius, respiratory system and other organs in birds (Nakamura and Meireles 2015).

So far, only four bird specific species of *Cryptosporidium* have been described: *Cryptosporidium meleagridis* Slavin, 1955; *Cryptosporidium baileyi* Current, Upton et Haynes, 1986; *Cryptosporidium galli* Pavlásek, 1999; and *Cryptosporidium avium* Holubová, Sak, Horčičková, Hlásková, Květoňová, Menchaca, McEvoy et Kváč, 2016 (Slavin 1955, Current et al. 1986, Ryan et al. 2003a, Holubová et al. 2016). These species infect a broad spectrum of birds, although they differ in their host range and site of infection; *C. meleagridis* also causes disease in humans (McLauchlin et al. 2000, Cama et al. 2003). Besides these four species, 13 *Cryptosporidium* genotypes have been described in birds worldwide, including avian genotypes I–VI, black duck genotype, Eurasian woodcock genotype and goose genotypes I–V (Nakamura and Meireles 2015, Chelladurai et al. 2016).

Additionally, the major human pathogenic species *C. hominis* Morgan-Ryan, Fall, Ward, Hijjawi, Sulaiman, Fayer, Thompson, Olson, Lal et Xiao, 2002 and *C. parvum* Tyzzer, 1912 and the artiodactyl and rodent species *C. andersoni* Lindsay, Upton, Owens, Morgan, Mead et Blagburn, 2000 and *C. muris* Tyzzer, 1907 have been detected in birds (Graczyk et al. 1998, Zhou et al. 2004, Ng et al. 2006, Qi et al. 2014).

Although cryptosporidiosis is one of the most prevalent parasitic infections in domestic, captive and wild birds worldwide, research on cryptosporidia in birds has lagged well behind that in mammals (Kváč et al. 2014). A major gap in our understanding of the diversity of cryptosporidia in birds is lack of prevalence and, in particular, genotyping data from the African continent (Bezuidenhout et al. 1993, Penrith et al. 1994, Soltane et al. 2007, Berrilli et al. 2012, Baroudi et al. 2013). In the present study, we determined the occurrence of *Cryptosporidium* spp. in domesticated and wild birds in Algeria using molecular tools, including subtype identification of *C. meleagridis*. Additionally, the role of wild birds as natural source of cryptosporidia for farmed birds was evaluated.

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MATERIALS AND METHODS

Samples

A total of 345 faecal samples from juvenile and adult domestic and wild birds were collected at poultry farms, rural and urban areas, and a zoo at the Bourdj Bou Arreridj Setif and Algiers province of Algeria (Table 1). At poultry farms, chicken and turkey broilers were kept on soil and laying hens were kept in hatcheries/cages. Birds from the zoo were housed in an aviary with a concrete floor or, in the case of ostriches, were kept on soil. Captive birds from urban areas were kept in bird cages. Faecal samples of wild and captive birds and poultry were obtained directly from the ground immediately after defecation. Cloacal contents of farmed birds were collected after necropsy. Each sample was placed into an individual sterile plastic container without fixative, transported to the laboratory in a cool box and stored at 4 °C until processing. All samples were screened for the presence of oocysts of cryptosporidia using the aniline-carbol-methyl violet staining method (Miláček and Vítovec 1985). Faecal consistency was noted at the time of sampling. The infection intensity was determined from the microscopic examination as number of oocysts per gram (OPG) according to Kváč et al. (2007).

Molecular study

DNA was extracted from 200 mg of faeces by bead disruption for 60 s at 5.5 m/s using 0.5 mm glass beads in a Fast Prep® 24 Instrument (MP Biomedicals, Santa Ana, CA, USA) followed by isolation/purification using a commercially available kit in accordance with the manufacturer's instructions (PSP Spin stool DNA Kit, STRATEC Molecular GmbH, Birkenfeld, Germany). Purified DNA was stored at -20 °C prior to being used for PCR analysis. A nested PCR approach was used to amplify a region of the small subunit of rRNA gene (SSU; ~ 830 bp; Xiao et al. 1999, Jiang et al. 2005) and actin (~ 1,066 bp; Sulaiman et al. 2002) in all samples. The 60 kDa glycoprotein (gp60; ~ 830 bp; Alves et al. 2003, Li et al. 2014, Guo et al. 2015) was amplified in positive samples on the basis of results of SSU and actin genotyping. Negative and positive controls (*C. parvum*) were included in all PCR sets. Purified secondary products (QIAquick® Gel Extraction Kit, Qiagen, Hilden, Germany) were sequenced in both directions with an ABI 3130 genetic analyser (Applied Biosystems, Foster City, CA, USA) using the secondary PCR primers.

The nucleotide sequences of each gene obtained in this study were edited using ChromasPro 1.7.5 software (Technelysium Pty Ltd., South Brisbane, Australia) and aligned with each other and with reference sequences from GenBank using MAFFT version 7 online server with automatic selection of alignment mode (http://mafft.cbrc.jp/alignment/software/). Phylogenetic analyses were performed and best DNA phylogeny models were selected using the MEGA6 software (Tamura et al. 2013). Phylogenetic trees with bootstrap support were inferred by maximum likelihood (ML) and maximum parsimony methods. The nucleotide sequences obtained in this study have been deposited in GenBank under accession numbers KY352474–KY352489.

Statistical analyses

A two-sample z-test for proportions (independent groups) was used to assess relationships between parasite detection (*Cryptosporidium* spp.) and presence or absence of signs of diarrhoea. All computations were performed with R 2.15.1.

RESULTS

Microscopical examination revealed the presence of oocysts of cryptosporidia in 22 of 345 faecal samples, with an infection intensity ranging from 1,000–4,000 OPG. Targeting the SSU and actin genes, *Cryptosporidium*-specific DNA was detected in all microscopy-positive samples and in nine samples that were microscopy-negative (Table 1). The infection rate of cryptosporidia varied among regions and farms. The prevalence at commercial chicken and turkey farms reached up to 9–69% and 13%, respectively (Table 1). Twelve examined animals suffered from diarrhoea at the time of sampling. However, only one of them was *Cryptosporidium*-positive (Table 1); thus, the presence of diarrhoea was not associated with *Cryptosporidium* infection (p value = 0.1871).

ML analysis of SSU and actin sequences revealed two distinct clusters among isolates of cryptosporidia from birds in the present study. Fourteen isolates clustered with *C. meleagridis*, sharing 100% sequence identity with *C. meleagridis* isolates with accession nos. AF112574 and AF382351. The remaining 17 isolates belonged to *C. baileyi*, sharing 100% sequence identity with accession nos. AF093495 and EU741853. Analysis of the gp60 gene of *C. meleagridis* isolates showed the presence of subtypes IIIgA22G3R1, in a wild goose (n = 1) and chicken broilers (n = 10), and IIIgA23G2R1 in turkey (n = 2) and chicken broilers (n = 1; Table 1). The gp60 gene of *C. baileyi* was not amplified by any of the used sets of primers. *Cryptosporidium meleagridis* and *C. baileyi* were found only on commercial farms and in wild birds. Cryptosporidia were not detected in traditionally bred poultry in rural areas.

DISCUSSION

In Algeria, only Baroudi et al. (2013) described the occurrence of *Cryptosporidium meleagridis* and *C. baileyi* in farmed poultry, whereas the occurrence of cryptosporidia in wild birds has not yet been studied. Therefore, the present study provides the first data on the prevalence of *Cryptosporidium* spp. in wild birds in Africa. *Cryptosporidium baileyi* is considered the most common avian species of *Cryptosporidium* worldwide and it has the broadest host range (Nakamura and Meireles 2015). In the present study, *C. baileyi* was detected in mallard, ostriches and chicken broilers. Whereas *C. baileyi* has been reported frequently in ducks and chickens worldwide (Morgan et al. 2001, Ryan et al. 2003b, Chvala et al. 2006, Huber et al. 2007), the only previous reports in ostriches have been from China and the Czech Republic (Ryan et al. 2003b, Wang et al. 2011). The overall prevalence of *C. baileyi* in ostriches in the present study reached 31%, which is comparable to the data by Wang et al. (2011) who reported prevalence of 1–29%, and lower than the 60% prevalence in farmed ostriches and rheas originating from Belgium, France, Netherlands, Portugal and Spain (Gordo et al. 2002). In contrast to the report of Wang et al. (2011), who did not detect

Table 1. A survey of *Cryptosporidium meleagridis* Slavin, 1955 and *C. baileyi* Current, Upton et Haynes, 1986 in faecal samples of domestic, captive and wild birds based on microscopic and molecular examination.

	Area	Host			Age	Screened samples/MIC/PCR positive	Molecular characterisation			Diarrhoea
							SSU	actin	gp60	
Wild birds	Rural	*Anser anser* (Linnaeus) (greylag goose)			A	4/0/0	-	-	-	-
						2/1/1	*C. meleagridis*	*C. meleagridis*	IIIgA22G3R1	-
						2/0/0	-	-	-	-
						3/0/0	-	-	-	-
		Anas platyrhynchos Linnaeus (mallard)			A	5/1/1	*C. baileyi*	*C. baileyi*	ND	1
					A	4/0/0	-	-	-	-
					A	14/0/0	-	-	-	-
					A	8/0/0	-	-	-	-
		Ciconia ciconia (Linnaeus) (white stork)			A	25/0/0	-	-	-	-
Captive birds	Zoo	*Dromaius novaehollandiae* (Latham) (emu)			A	3/0/0	-	-	-	-
						3/0/0	-	-	-	-
		Balearica regulorum (Bennett) (grey crowned-crane)			A	4/0/0	-	-	-	-
		Numida meleagris (Linnaeus) (helmeted guineafowl)			A	2/0/0	-	-	-	-
		Anas platyrhynchos Linnaeus (mallard)			A	6/0/0	-	-	-	-
						2/0/0	-	-	-	-
		Eclectus roratus (Müller) (eclectus parrot)			A	5/0/0	-	-	-	-
						1/0/0	-	-	-	-
		Pavo sp. (peacock)			A	3/0/0	-	-	-	-
						6/0/0	-	-	-	-
		Columba livia f. *domestica* Gmelin (domestic pigeon)			A	2/0/0	-	-	-	-
		Phasianus colchicus Linnaeus (ring-necked pheasant)			A	2/0/0	-	-	-	-
						3/0/0	-	-	-	-
		Struthio camelus Linnaeus (ostrich)			A	13/4/4	*C. baileyi*	*C. baileyi*	ND	-
						2/0/0	-	-	-	-
	Urban	*Melopsittacus undulatus* (shaw) (Budgerigar)			J	2/0/0	-	-	-	-
		Serinus canaria (Linnaeus) (canary)			J	6/0/0	-	-	-	-
						5/0/0	-	-	-	-
		Carduelis carduelis (Linnaeus) (European goldfinch)			J	10/0/0	-	-	-	-
Domestic birds	Rural	*Gallus gallus* f. *domestica* (Linnaeus) (hen)	commercial breed	chicken broiler	2–3 weeks	6/0/0	-	-	-	-
					3–4 weeks	11/0/0	-	-	-	3
					3–5 weeks	16/11/11	*C. baileyi*	*C. baileyi*	ND	-
					4 weeks	7/0/0	-	-	-	7
					5 weeks	8/1/1	*C. baileyi*	*C. baileyi*	ND	-
					5–6 weeks	11/1/1	*C. meleagridis*	*C. meleagridis*	IIIgA23G2R1	-
					5–6 weeks	8/0/0	-	-	-	-
					5–7 weeks	9/0/0	-	-	-	-
					8 weeks	16/2/10	*C. meleagridis*	*C. meleagridis*	IIIgA22G3R1	-
				laying hen	2 weeks	16/0/0	-	-	-	-
					A	13/0/0	-	-	-	-
						12/0/0	-	-	-	-
				broiler hen	A	8/0/0	-	-	-	-
						7/0/0	-	-	-	-
			traditional breed	hen	A	3/0/0	-	-	-	-
						2/0/0	-	-	-	-
						5/0/0	-	-	-	-
						2/0/0	-	-	-	1
		Meleagris gallopavo f. *domestica* Linnaeus (turkey)	commercial breed	turkey broiler	4–7 weeks	19/0/0	-	-	-	-
					14–16 weeks	15/1/2	*C. meleagridis*	*C. meleagridis*	IIIgA23G2R1	-
			traditional breed		A	4/0/0	-	-	-	-
Total						345/22/31				12

J – juvenile; A – adult; MIC – microscopy; PCR – Polymerase Chain Reaction; ND – not detected.

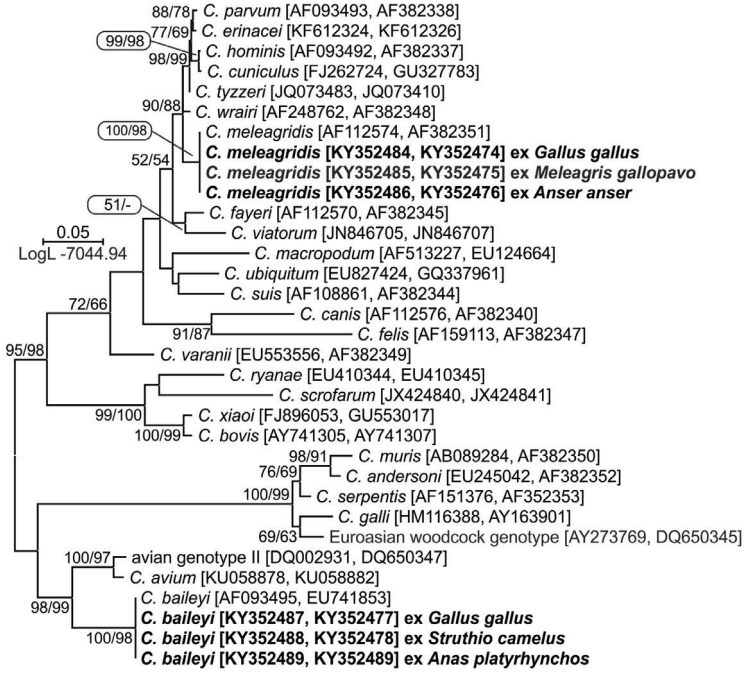

Fig. 1. Concatenated maximum likelihood tree (model Tamura 3-parameter G + I) based on partial small subunit ribosomal RNA and actin gene sequences of species and genotypes of *Cryptosporidium* Tyzzer, 1910, including those detected in the present study. Newly obtained sequences are bolded. Numbers at the nodes represent the bootstrap values (maximum likelihood/maximum parsimony; 1,000 replicates) gaining more than 50% support. Branch length scale bar indicates number of substitution per site.

Cryptosporidium in ostriches older than 12 months of age, we reported a high prevalence in adult ostriches. Although there have been many reports concerning *Cryptosporidium* infections in ostriches, the knowledge of species identity and their host and age specificity is far from clear. In contrast, the biological properties of *C. baileyi* in chicken is well known (Robertson et al. 2014). In Algeria, we detected a high prevalence of this species on a commercial farm, where 69% of screened birds were positive, and the overall prevalence of *C. baileyi* in this study varied from 9 to 69%. Our findings are consistent with the reported prevalence of *C. baileyi* in flocks of chicken broilers in the USA (10–60%) (Goodwin et al. 1996), Morocco (14–100%) (Kichou et al. 1996) and China (7%) (Wang et al. 2014).

Cryptosporidium meleagridis has been found at a high prevalence (29%) in turkeys aged over 4 weeks in Algeria (Baroudi et al. 2013). In contrast, we detected the same species in only 13% of 14–16 weeks old turkey broilers kept on the commercial farms, which is similar to the data reported by McEvoy and Giddings (2009), who found 3–11% of 4–9 week-old poults infected with *C. meleagridis*. Similarly to turkey flocks, infection of chickens with *C. meleagridis* varied. In the present study, *C. meleagridis* was detected in 9–63% of chicken broilers. Wang et al. (2014) reported a much lower prevalence (less than 2%) of *C. meleagridis* in chickens. In contrast, other authors reported *C. meleagridis* in 35–44% of turkeys (Pavlásek 1994, Gharagozlou et al. 2006, Baroudi et al. 2013). These results show high variability among breeds worldwide, but different methodological approaches of surveys, differences in hygiene and host age likely play important role. In contrast to previous studies that frequently reported clinical cryptosporidiosis in turkeys and chickens (Goodwin 1988, Goodwin et al. 1988, Baroudi et al. 2013, Nakamura and Meireles 2015), infection with *C. meleagridis* was not associated with diarrhoea and mortality in the present study.

The importance of cryptosporidiosis in commercial poultry production has not yet been determined because few studies have examined the relationship between natural infection by *Cryptosporidium* spp. and production losses (Nakamura and Meireles 2015). The common occurrence of *C. meleagridis*, mainly in domestic birds, may have an impact on public health. *Cryptosporidium meleagridis* is the third most common human-pathogenic species in both developing and developed countries (Cama et al. 2008). All isolates of *C. meleagridis* detected in the present study belonged to family IIIg, which has been previously detected in birds and humans (Abal-Fabeiro et al. 2013, Baroudi et al. 2013, Stensvold et al. 2014). Two subtypes of *C. meleagridis*, IIIgA23G2R1 and IIIgA22G3R1, have not been reported previously from domestic chickens, turkeys and wild goose. The presence of subtype IIIgA22G3R1 in wild and farmed birds in this study suggests circulation

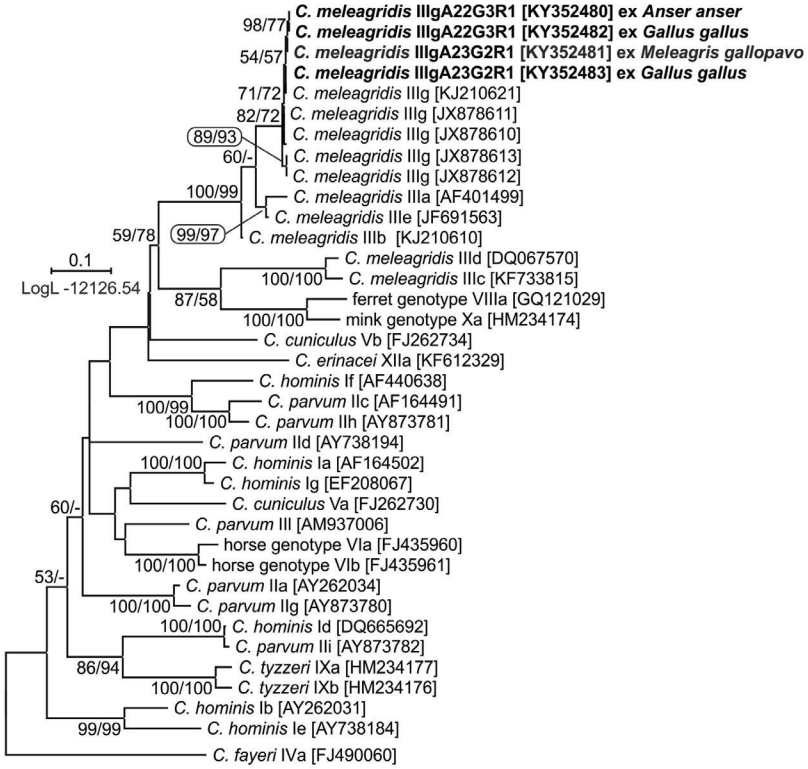

Fig. 2. Maximum likelihood tree (model General Time Reversible G + I) based on partial sequences of gp60 gene of species and genotypes of *Cryptosporidium* Tyzzer, 1910, including *Cryptosporidium meleagridis* Slavin, 1955 detected in the present study. Newly obtained sequences are bolded. Numbers at the nodes represent the bootstrap values (maximum likelihood/maximum parsimony; 1,000 replicates) gaining more than 50% support. Branch length scale bar indicates number of substitution per site.

of parasites between wild and domestic animals. Due to the lack of gp60 subtyping on human cryptosporidiosis in North Africa, it is not known if the *C. meleagridis* subtypes reported in the present study are associated with human disease, although infections by this species has been previously reported in Tunisia (Essid et al. 2008, Rahmouni et al. 2014).

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7.4 Příloha IV

Host specificity and age-dependent resistance to *Cryptosporidium avium* infection in chickens, ducks and pheasants.

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Host specificity and age-dependent resistance to *Cryptosporidium avium* infection in chickens, ducks and pheasants

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ABSTRACT

Host- and age-specificity of *Cryptosporidium avium* were studied in 1-, 21- and 365-day-old chickens (*Gallus gallus*), domestic ducks (*Anas platyrhynchos*) and ring-necked pheasants (*Phasianus colchicus*) under experimental conditions. *Cryptosporidium avium* was not infectious for ring-necked pheasants, but it was infectious for ducks and chickens at all age categories. The course of infection in ducks did not differ among age categories, but 365-day-old chickens had less severe infections than 1- and 21-day-old chickens. The patent period in chickens and ducks was > 30 DPI, but ducks started to shed oocysts of *C. avium* earlier (5–6 DPI) and at a lower intensity (accumulated value of infection intensity of 58,000–65,000 OPG) than chickens (9–11 DPI and accumulated value of infection intensity of 100,000–105,000 OPG). Experimentally infected birds showed no clinical signs of cryptosporidiosis.

1. Introduction

The genus *Cryptosporidium* comprises protist parasites that infect epithelial cells of the gastrointestinal tract of all vertebrates (Ryan and Xiao, 2014). Additionally, some species and genotypes of the genera *Cryptosporidium* infect the bursa of Fabricius and other organs in birds (Nakamura and Meireles, 2015). The genetic diversity of *Cryptosporidium* spp. has been well studied, and several genotypes have been described in birds, most of which have a limited host range (Kváč et al., 2014a). For example, duck genotype and goose genotypes I-IV have been found exclusively in the order Anseriformes (Cano et al., 2016; Morgan et al., 2001; Zhou et al., 2004), while avian genotype II has been found exclusively in Psittaciformes (Ng et al., 2006).

Biological characteristics have been less well studied in bird-adapted *Cryptosporidium* spp. than in species infecting mammals. *Cryptosporidium baileyi*, *C. meleagridis* and *C. galli* have been reported in birds belonging to several different avian orders (Kváč et al., 2014a; Robertson et al., 2014). Experimental infectivity studies have shown that the susceptibility of birds to *C. baileyi* and *C. galli* can vary with age (Pavlásek, 2001; Sreter and Varga, 2000; Sreter et al., 1995).

Cryptosporidium avium is a recently described species that has been reported to naturally infect budgerigars (*Melopsittacus undulates*), cockatiels (*Nymphicus hollandicus*), Fischer's lovebirds (*Agapornis fischeri*), major Mitchell's cockatoos (*Lophochroa leadbeateri*), red-crowned parakeets (*Cyanoramphus novaezelandiae*), rosy-faced lovebirds (*Agapornis roseicollis*) and turquoise-fronted amazons (*Amazona aestiva*) from the order Psittaciformes (Abe and Makino, 2010; Curtiss et al., 2015; Li et al., 2016; Nakamura and Meireles, 2015; Qi et al., 2011; Zhang et al., 2015). Wang et al. (2014) also described *C. avium* in chickens (*Gallus gallus*) in the order Galliformes. All cases of natural infections in birds have been from adults, but Holubová et al. (2016) demonstrated that *C. avium* can infect 6-month-old chickens under experimental conditions. Additionally, *C. avium* was associated with colitis and cystitis in green iguanas (*Iguana iguana*) in the order Squamata (Kik et al., 2011).

We recently detected natural *C. avium* infections in ducks from the order Anseriformes (unpublished), suggesting that this species can infect multiple avian orders, similar to *C. baileyi* and *C. galli*. Therefore, the present study aimed at determining the susceptibility of birds of different ages from the orders Galliformes and Anseriformes to experimental infection by *C. avium*.

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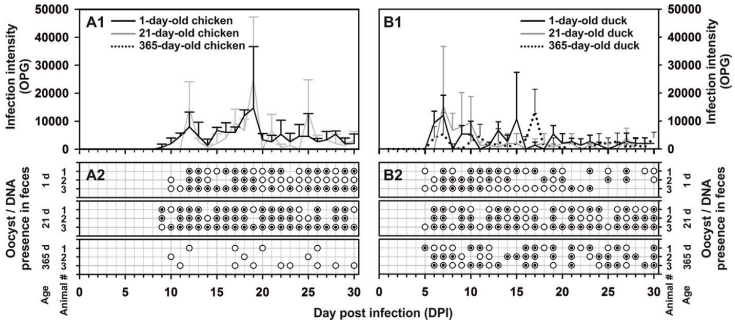

Fig. 1. Course of infection caused by *Cryptosporidium avium* based on microscopical and molecular examination of faeces of **A)** domestic chickens (*Gallus gallus*) and **B)** domestic ducks (*Anas platyrhynchos*) inoculated with 10,000 oocysts. **1)** Oocyst shedding per gram of faeces is expressed as the mean from examined animals with standard errors. **2)** Circles indicate detection of specific DNA. Black dots indicate microscopic detection of oocysts.

Table 1
Course of infection in caused by *Cryptosporidium avium* in domestic chickens (*Gallus gallus*), domestic ducks (*Anas platyrhynchos*) and ring-necked pheasants (*Phasianus colchicus*) and evaluated on the basis of the length of the prepatent and patent period, average number of excreted oocyst during patent period per animal (AUC), maximum infection intensity concentration (Cmax) and maximum infection intensity time (tmax).

Host (scientific name)	Age category	Prepatent period (day)	Patent Period (day)	Course of infection		
				AUC (OPG)	Cmax (OPG)	Tmax (day)
Chickens (*Gallus gallus*)	1-day	10–12	> 30	100,000	38,000	19
	21-days	9–10	> 30	105,000	22,000	19
	365-days	11–12	> 30	–	–	–
domestic ducks (*Anas platyrhynchos*)	1-day	5–6	> 30	60,000	22,000	10
	21-days	5–6	> 30	65,000	18,000	10
	365-days	5–6	> 30	58,000	18,000	11
ring-necked pheasants (*Phasianus colchicus*)	1-day	Insusceptible hosts				
	21-days					
	365-days					

2. Material and methods

Oocysts of *C. avium* were obtained from the intestinal contents of five naturally infected and hunted adult wild ducks (*Anas platyrhynchos*) in Plzeň, the Czech Republic. The identity of *C. avium* has been confirmed by PCR/sequencing of the *Cryptosporidium* small-subunit rRNA gene (SSU; below). All intestinal contents (15–30 g from each animal) were pooled and the oocysts were purified using a cesium chloride gradient (Arrowood and Donaldson, 1996) and stored in PBS at 4 °C. Oocysts in the inoculum were genotyped using the molecular methods described below, enumerated using a haemocytometer, and examined for viability using an assay previously described by Sauch et al. (1991). Groups of naïve 1-, 21- and 365-day-old chickens (*Gallus gallus*), domestic ducks (*Anas platyrhynchos*) and ring-necked pheasants (*Phasianus colchicus*), each comprising three animals, were used in the study. All animals were hatched and kept under laboratory conditions to prevent infection by *Cryptosporidium* prior to inoculation. The study was conducted in accordance with Act No. 246/1992 Coll. of the Czech Republic. The research protocols (Protocols No. 071/2010 and 114/2013) were approved by the Committee for Animal Welfare of the Biology Centre, Czech Academy of Science and the Veterinary administration authorities. Animals of each host and age category were housed separately in cages with raised bottom grids, to avoid re-infection, and were fed with sterile commercial poultry feed and sterile water *ad libitum*. Prior to experiments, faecal samples from all animals were screened for the presence of *Cryptosporidium* spp. using the parasitological and molecular methods described below. Each animal was inoculated orally with 10,000 purified, viable oocysts suspended in 200 µl of distilled water. Faeces from all animals were sampled daily from 3 to 30 days post infection (DPI), when the experiment was terminated. Infection intensity was reported as the number of oocysts per gram (OPG) of faeces, as previously described Kváč et al. (2007) following staining of faecal smears using the method described by Miláček and Vítovec (1985). DNA was extracted from faecal samples using the Exgene Stool SV mini kit (GeneAll, Seoul, Korea) following homogenization by glass beads, and partial sequences of SSU (Jiang et al., 2005), actin (Sulaiman et al., 2002) and 70-kDa heat shock protein (HSP70; Sulaiman et al., 2000) genes were amplified using a nested PCR approach. At least three amplicons were sequenced directly in both directions using an ABI3130 sequencer analyser (Applied Biosystems, Forster City, CA, USA). Obtained nucleotide sequences were compared with each other and with reference sequences using BioEdit 7.0.5.3 (Hall, 1999). All statistical analyses were carried out using the programming environment R 2.15.0. The accumulated value of infection intensity was calculated as area under the curve (AUC), tmax (time at maximal intensity) and Cmax (maximal intensity) through the classical trapezoidal rule. Differences in the course of infection among groups of experimentally infected animals were tested using the *t*-test. Because of planned multiple comparisons between particular groups, evaluation of significance was carried out after Bonfferroni adjustment. Differences were considered significant when $P < 0.05$.

3. Results and discussion

Here, using microscopic and PCR analysis of faecal samples for oocysts and DNA, respectively, we show that *C. avium* is not infectious for ring-necked pheasants, a Galliformes species that is susceptible to infection by *C. baileyi*, *C. galli* and *C. meleagridis* (Ashraf et al., 2015; Máca and Pavlásek, 2016; Pavlásek and Kozakiewicz, 1989; Randall, 1986; Whittington and Wilson, 1985), but it is infectious for three age categories of chickens and ducks.

The onset of shedding (oocysts or DNA) did not differ ($P > 0.05$)

among age categories of either susceptible host (Fig. 1), but there were some differences in the course of infection. The prepatent period in chickens (9–11 DPI) was longer than that in ducks (5–6 DPI; $P < 0.05$) and similar to that previously reported in budgerigars (*Melopsittacus undulatus*) (Holubová et al., 2016). The patent period in all age categories of chickens and ducks was at least 30 DPI, the point at which the experiment was terminated. One- and 21-day old chickens shed similar numbers of oocysts ($P > 0.05$), which were detectable by microscopy throughout the patent period (Table 1). Chickens infected at 365-days-old failed to shed detectable oocysts at any time during the infection, although DNA was detected intermittently in faecal samples. Sreter et al. (1995) found that older chickens shed fewer oocysts of *C. baileyi*, the avian-adapted species that is most closely related to *C. avium*, than younger chickens. Also, Pavlásek (2001) showed that *C. galli* oocysts were infectious for 9-day-old but not 40-day-old chickens under experimental conditions.

In contrast to chickens, ducks of different ages (1, 21- and 365-days-old) shed similar number of *C. avium* oocysts throughout the patent period ($P > 0.05$), although the shedding intensity in ducks was about half that 1- and 21-day-old chickens (Fig. 1, Table 1). The effect of age on host susceptibility to *Cryptosporidium* infection is poorly understood, and it varies by host and parasite species. Similarly to ducks infected with *C. avium*, the susceptibility of mice to *C. tyzzeri* does not vary by age (Ren et al., 2012). In contrast, mice (BALB/c, *Mus musculus*) and gerbils (*Meriones unguiculatus*) have decreasing susceptibility to *C. parvum* with increasing age (Baishanbo et al., 2005; Kváč et al., 2009; Tarazona et al., 1998). One factor that could contribute to decreasing susceptibility to *Cryptosporidium* with increasing age is the maturation of the immune response (Colditz et al., 1996); however, immune development is not the only contributing factor. Naïve piglets with an immature immune system are resistant to *C. scrofarum* infection and become susceptible to the infection with increasing age (Kváč et al., 2014b), indicating that some other factors could affect age dependent susceptibility.

All isolates from experimentally infected ducks and chickens had SSU, actin and HSP70 sequences that were identical to those from the *C. avium* isolate used in the inoculum (GenBank accession numbers KU0588878, KU058882 and KU058886 for SSU, actin and HSP70 nucleotide sequences, respectively). Consistent with most reports describing natural and experimental infections with *C. avium* (Holubová et al., 2016; Ng et al., 2006), none of the infected birds showed clinical signs of cryptosporidiosis. This is in contrast to the report of lethargy, anorexia, and cloacal prolapse in a 7-year-old Major Mitchell's cockatoo (*Lophochroa leadbeateri*) infected with *C. avium*.

The finding that ducks were susceptible to experimental infection by *C. avium* was expected following our detection of natural *C. avium* infections in 11/350 (3.1%) ducks from the Czech Republic (unpublished). The occurrence of natural infections and the course of the experimental infection, which includes a short prepatent period (5–6 DPI), long patent period (> 30 DPI), and shedding of detectable oocysts by birds in all age categories, suggests that ducks are a significant host for *C. avium*. Despite our considerable knowledge of the genetic diversity of *Cryptosporidium*, we still do not fully understand the drivers of host specificity. These findings emphasize that biological properties of *Cryptosporidium* species cannot be accurately predicted based on phylogenetic relatedness, and therefore experimental infectivity studies are critically important.

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7.5 Příloha V

Cryptosporidium proventriculi sp. n. (Apicomplexa: Cryptosporidiidae) in Psittaciformes birds.

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Cryptosporidium proventriculi sp. n. (Apicomplexa: Cryptosporidiidae) in Psittaciformes birds

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Abstract

Cryptosporidiosis is a common parasitic infection in birds that is caused by more than 25 *Cryptosporidium* species and genotypes. Many of the genotypes that cause avian cryptosporidiosis are poorly characterized. The genetic and biological characteristics of avian genotype III are described here and these data support the establishment of a new species, *Cryptosporidium proventriculi*. Faecal samples from the orders Passeriformes and Psittaciformes were screened for the presence of *Cryptosporidium* by microscopy and sequencing, and infections were detected in 10 of 98 Passeriformes and in 27 of 402 Psittaciformes. *Cryptosporidium baileyi* was detected in both orders. *Cryptosporidium galli* and avian genotype I were found in Passeriformes, and *C. avium* and *C. proventriculi* were found in Psittaciformes. *Cryptosporidium proventriculi* was infectious for cockatiels under experimental conditions, with a prepatent period of six days post-infection (DPI), but not for budgerigars, chickens or SCID mice. Experimentally infected cockatiels shed oocysts more than 30 DPI, with an infection intensity ranging from 4,000 to 60,000 oocysts per gram (OPG). Naturally infected cockatiels shed oocysts with an infection intensity ranging from 2,000 to 30,000 OPG. *Cryptosporidium proventriculi* infects the proventriculus and ventriculus, and oocysts measure 7.4 × 5.8 μm. None of the birds infected *C. proventriculi* developed clinical signs.

Keywords: *Cryptosporidium* avian genotype III; Experimental infections; Oocyst size; PCR; Prevalence; Taxonomy

Introduction

Cryptosporidium parasites belong to the phylum Apicomplexa and infect the gastrointestinal tract of a broad range of vertebrate species (Smith et al. 2007; Sreter and Varga 2000). Currently, 41 species of *Cryptosporidium* are recognized in

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fish, amphibians, reptiles, birds, and mammals (Kváč et al. 2014; Liu et al. 2013; Qi et al. 2011). In contrast, more than 70 genotypes have been described that await the morphological and biological characterization necessary to describe them as separate species (Chalmers et al. 2018; Ryan et al. 2014; Xiao et al. 1999; Xiao and Ryan 2004).

To date, four avian *Cryptosporidium* spp. have been described in birds: *Cryptosporidium meleagridis* (Slavin 1955), *Cryptosporidium baileyi* (Current et al. 1986), *Cryptosporidium galli* (Ryan et al. 2003b) and *Cryptosporidium avium* (Holubová et al. 2016). In addition, 21 *Cryptosporidium* genotypes have been identified in more than 30 avian species worldwide (Ryan 2010), and these appear to be avian specific. They include avian genotypes I–IV and VI–IX (Helmy et al. 2017), goose genotypes I–IV (Zhou et al. 2004) and Id (Cano et al. 2016), duck genotypes I and II (Jellison et al. 2004; Morgan et al. 2001; Zhou et al. 2004) and duck genotype b (Cano et al. 2016), Eurasian Woodcock genotype (Ryan et al. 2003a), Finch genotypes I–III (Morgan et al. 2000) and YS-2017 genotype from owls (Makino et al. 2018). Mammal-specific *Cryptosporidium* spp., including *C. andersoni*, *C. hominis*, *C. muris*, *C. parvum*, *C. canis*, have been reported rarely in birds (Ferrari et al. 2018; Helmy et al. 2017; Nakamura et al. 2009; Ng et al. 2006; Oliveira et al. 2017; Qi et al. 2014; Santín et al. 2004).

Cryptosporidium meleagridis, *C. baileyi* and *C. galli* infect the intestine, lungs, and proventriculus, respectively, and have been found in several avian orders, suggesting low host specificity in avian hosts (Baroudi et al. 2013; Jellison et al. 2004; Li et al. 2015; Ng et al. 2006; Wang et al. 2014). In contrast, *C. avium*, which is closely related to *C. baileyi* and infects the same site, appears to be restricted to hosts in the order Psittaciformes (Holubová et al. 2016; Slavin 1955). Most avian *Cryptosporidium* genotypes have been detected in a small number of avian hosts, but the data are insufficient to support the conclusion that they have a narrow host range, similar to *C. avium* (Nakamura and Meireles 2015). The present study aimed to address this deficiency for *Cryptosporidium* avian genotype III, which infects the proventriculus and ventriculus of birds belonging to five orders of class Aves (Table 1). We use experimental infections to confirm that avian genotype III has a different host range than *C. galli*, and we also show that it differs from *C. galli* in other biological characteristics, oocyst morphology, and nucleotide sequence at multiple loci. Based on these and other reported data, we propose that *Cryptosporidium* avian III be recognized as a new *Cryptosporidium* species, *Cryptosporidium proventriculi* sp. n.

Material and Methods

Specimens studied

Faecal samples from pet birds owned by private breeders in the Czech Republic (n = 10), Slovakia (n = 2) and Poland (n = 3) were sampled. Each sample was collected from the floor of the bird cage immediately after defecation and placed into a separate plastic tube without fixative. The faecal consistency (loose if it took the form of the container and solid if it maintained its original shape) was noted at the time of sampling. Each animal was sampled only once. All animals were screened without previous knowledge of parasitological status. A total of 402 and 98 samples were obtained from birds in the order Psittaciformes and Passeriformes, respectively (Table 2). All samples were stored at 4 °C until used for further analysis.

Origin of specimens for transmission studies

Isolates of *Cryptosporidium proventriculi* sp. n. (previously known as *Cryptosporidium* avian genotype III) were obtained from four naturally infected adult cockatiels (*Nymphicus hollandicus*) (Czech Republic). *Cryptosporidium proventriculi* sp. n. oocysts from positive birds were pooled and used to infect an adult cockatiel, which was negative for *Cryptosporidium* DNA by PCR and was sourced from a breeder that had no *Cryptosporidium*-positive birds. Oocysts from the infected cockatiel were purified using caesium chloride gradient centrifugation (Arrowood and Donaldson 1996) and used for oocyst morphometry and infectivity studies (see Transmission studies).

Parasitological examination and oocyst preparation

All animal faeces were screened for *Cryptosporidium* oocysts using faecal smears stained with aniline-carbol-methyl violet (ACMV) (Miláček and Vítovec 1985). Faecal specimens were collected daily and stored in a 2.5% potassium dichromate solution at 4–8 °C.

Cryptosporidium oocysts from cockatiels were purified using caesium chloride gradient centrifugation prior to morphometric analyses and transmission studies (Kilani and Sekla 1987). The viability of oocysts was examined using propidium iodide (PI) staining by a modified assay of Sauch et al. (1991). Briefly, examined oocysts were washed in distilled water (DW; 10,000 oocysts in 10 μl) and mixed with 0.1 μl of PI (1% solution, SIGMA). After 30 min of incubation at room temperature in the dark, the oocysts were washed twice with DW. Oocyst viability was examined using fluorescence microscopy (filter 420 nm, Olympus IX70). Oocysts with red fluorescence were considered to be dead, and those without fluorescence were considered viable.

Oocyst morphometry

The morphology and morphometry of *Cryptosporidium proventriculi* sp. n. oocysts were examined using differential interference contrast (DIC) microscopy, brightfield microscopy following ACMV and modified Ziehl-Neelsen (ZN; Henriksen and Pohlenz 1981) staining and fluorescence microscopy following labelling with genus-specific FITC-

Table 1. Occurrence of *Cryptosporidium proventriculi* sp. n. (formerly known as *Cryptosporidium* avian genotype III) in birds from orders Psittaciformes[1], Passeriformes[2], Piciformes[3], Charadriiformes[4] and Anseriformes[5] demonstrated on the basis of molecular tools amplifying partial sequences of *Cryptosporidium* small-subunit rRNA (SSU), actin and *Cryptosporidium* oocyst wall protein (COWP) genes.

Host (scientific name)	Country	Loci for genotyping [GenBank Acc. No.]	No. of screened/ positive	References
Blue-fronted parrot[1] (*Amazona aestiva*)	Brazil	SSU[b]	NS/1	Nakamura et al. (2014)
Barred parakeet[1] (*Bolborhynchus lineola*)	Brazil	SSU [MF462155]	34/1	Ferrari et al. (2018)
Cockatiel[1] (*Nymphicus hollandicus*)	Australia	SSU [DQ650343]	NS/3	Ng et al. (2006)
	Brazil	SSU [identical with GQ227480]	8/1	Nakamura et al. (2014)
		SSU [GQ227481]	64/1	Nakamura et al. (2009)
		SSU [GU074385-87]	NS/3	Gomes et al. (2012)
		SSU [identical with GQ227480]	70/9	Ferrari et al. (2018)
	China	SSU [HM116385]	39/2	Qi et al. (2011)
	India	SSU [KX668210]	NA	unpublished
	Japan	SSU [identical with AB694729]	10/1	Iijima et al. (2018)
		SSU [AB471645] COWP [AB471653] Actin [AB471659]	4/1	Abe and Makino (2010)
Forpus sp.[1]	Brazil	SSU [MF462156]	78/12	Ferrari et al. (2018)
Galah[1] (*Eolophus roseicapilla*)	Australia	Actin [DQ650349]	NS/1	Ng et al. (2006)
		SSU[b]	13/2	Nakamura et al. (2014)
Lovebird[1] (*Agapornis* sp.)	Brazil	SSU [identical with GQ227480]	14/3	Ferrari et al. (2018)
	USA	SSU [KJ661334]	18/2	Ravich et al. (2014)
Lilian's lovebird[1] (*Agaponis lilianae*)	Japan	SSU [identical with AB694729]	5/1	Iijima et al. (2018)
Neophema sp. [1]	Brazil	SSU[b]	91/1	Ferrari et al. (2018)
Pacific parrotlet[1] (*Forpus coelestis*)	Japan	SSU [identical with AB694729]	3/1	Iijima et al. (2018)
Peach-faced lovebird[1] (*Agapornis roseicollis*)	Brazil	SSU [GQ227480]	14/1	Nakamura et al. (2009)
	Japan	SSU [AB471641] Actin [AB471655]	37/13	Makino et al. (2010)
		SSU [identical with AB694729]	29/5	Iijima et al. (2018)
Red-rumped parrot[1] (*Psephotus haematonotus*)	Brazil	SSU*	21/1	Ferrari et al. (2018)
Sun parakket[1] (*Aratinga solstitialis*)	Australia	SSU [DQ650342]	NS/1	Ng et al. (2006)
Double-collared seedeater[2] (*Sporophila caerulescens*)	Brazil	SSU[b]	10/1	Nakamura et al. (2014)
Green winged saltator[2] (*Saltator similis*)	Brazil	SSU*	152/1	Nakamura et al. (2014)
Island canary[2] (*Serinus canaria*)	Brazil	SSU[b]	498/12	Camargo et al. (2018)
Java sparrow[2] (*Padda oryzivora*)	Brazil	SSU [GU074384]	NS/1	Gomes et al. (2012)
Red-billed blue magpie[2] (*Urocissa erythrorhyncha*)	China	SSU [HM116386]	1/1	Qi et al. (2011)
Rufous-collared sparrow[2] (*Zonotrichia capensis*)	Brazil	SSU*	NS/1	Nakamura et al. (2014)
Sporophila sp.[2]	Brazil	SSU*	NS/1	Nakamura et al. (2014)

Table 1 (*Continued*)

Host (scientific name)	Country	Loci for genotyping [GenBank Acc. No.]	No. of screened/ positive	References
Saffron toucanet[3] (*Pteroglossus bailloni*)	Brazil	SSU [KU885389]	2/1	Novaes et al. (2018)
Red-billed toucan[3] (*Ramphastos tucanus*)	Brazil	SSU [KU885388]	4/2	Novaes et al. (2018)
Toco toucan[3] (*Ramphastos toco*)	Brazil	SSU [KU885387]	28/5	Novaes et al. (2018)
Seagul[4] (*Chroicocephalus brunnicephalus* and *ridibundus*)	Thailand	SSU [identical with AB694729]	70/2	Koompapong et al. (2014)
Waterbird[5,a]	Spain	SSU [KT880495-97]	265/4	Cano et al. (2016)

NS not specified.
[a] bird species was not determined.
[b] based on the duplex real-time PCR targeting the SSU gene in *Cryptosporidium* avian genotype III.

conjugated antibodies (IFA; *Cryptosporidium* IF Test, Crypto cel, Cellabs Pty Ltd., Brookvale, Australia).

Morphology and morphometry were determined using digital analysis of images (Olympus cellSens Entry 2.1, Olympus Corporation, Shinjuku, Tokyo, Japan) collected using an Olympus Digital Colour camera DP73 microscope. Length and width of oocysts (n = 100) from experimentally infected cocktails were measured under DIC at 1000 × magnification and these measurements were used to calculate the length-to-width ratio. As a control, the morphometry of *C. baileyi* (n = 100) and *C. avium* (n = 50) from experimentally infected chickens (*Gallus gallus* f. *domestica*) and budgerigars (*Melopsittacus undulatus*), respectively, were measured by the same person using the same microscope. Photomicrographs of *C. proventriculi* sp. n. oocysts observed by DIC, ACMV, ZN and IFA were deposited as a photo type at the Institute of Parasitology, Biology Centre of the Czech Academy of Sciences, Czech Republic.

Molecular characterization

DNA was extracted from 200 mg of faeces, 10,000 purified oocysts, or 200 mg of tissue by bead disruption for 60 s at 5.5 m/s using 0.5 mm glass beads in a FastPrep® 24 Instrument (MP Biomedicals, CA, USA) followed by isolation/purification using a commercially available kit in accordance with the manufacturer's instructions (Exgene™ Stool DNA mini, GeneAll Biotechnology Co. Ltd, Seoul, Korea). Purified DNA was stored at −20 °C prior to being used for PCR. A nested PCR approach was used to amplify a partial region of the small ribosomal subunit rRNA (~830 bp; SSU; Xiao et al. 1999), actin (~1950 bp; Sulaiman et al. 2000), and 70 kilodalton heat shock protein genes (~515 bp; HSP70; Chelladurai et al. 2016). For the SSU fragment, primary amplification was employed with the primers 5′TTC TAG AGC TAA TAC ATG CG3′ and 5′CCC ATT TCC TTC GAA ACA GGA3′ followed by secondary amplification with the primers 5′GGA AGG GTT GTA TTT ATT AGA TAA AG3′ and 5′AAG GAG TAA GGA ACA ACC TCC A3′. For the actin fragment, primary amplification was employed with the primers 5′ATC RGW GAA GAA GWA RYW CAA GC3′ and 5′AGA ARC AYT TTC TGT GKA CAA T3′ followed by secondary amplification with the primers 5′CAA GCW TTR GTT GTT GAY AA3′ and 5′TTT CTG TGK ACA ATW SWT GG3′. For the HSP70 fragment, primary amplification was employed the primers 5′GCT CGT GGT CCT AAA GAT AA3′) and 5′ACG GGT TGA ACC ACC TAC TAA T3′ followed by secondary amplification with the primers 5′ACA GTT CCT GCC TAT TTC3′) and 5′GCT AAT GTA CCA CGG AAA TAA TC3′.

The primary PCR mixtures contained 2 μl of template DNA, 2.5 U of Taq DNA Polymerase (Dream Taq Green DNA Polymerase, Thermofisher Scientific, Waltham, MA, USA), 0.5 × PCR buffer (SSU) or 1 × PCR buffer (actin and HSP70; Thermofisher Scientific), 6 mM MgCl2 (SSU) or 3 mM MgCl2 (actin and HSP70), 200 μM each deoxynucleoside triphosphate, 100 mM each primer and 2 μL non-acetylated bovine serum albumin (BSA; 10 mg ml−1; New England Biolabs, Beverly, MA, USA) in 50 μl reaction volume. The secondary PCR mixtures were similar to those described above for the primary PCR, with the exception that 2 μl of the primary PCR product was used as the template, the MgCl2 concentration was 3 mM and no BSA was used. DNA of *C. parvum* and molecular grade water were used as positive and negative controls, respectively.

Secondary PCR products were detected by agarose gel electrophoresis, visualized by ethidium bromide staining and extracted using Gen Elute Gel Extraction Kit (Sigma, St. Louis, MO, USA). Purified secondary products were sequenced in both directions with an ABI 3130 genetic analyser (Applied Biosystems, Foster City, CA) using the secondary PCR primers and the BigDye1 Terminator V3.1

Table 2. *Cryptosporidium* species and genotypes from this study, detected by amplification of small subunit ribosomal rRNA (SSU), actin and 70 kDa Heat Shock Protein (HSP70) gene fragments in birds from the orders Psittaciformes and Passeriformes from the Czech Republic (CZK), Poland (POL) and Slovakia (SVK). Infection intensity of *Cryptosporidium* spp. is expressed as the number of oocysts per gram of faeces (OPG).

Order	Host (scientific name)	Number of screened/ positive	ID of positive animal	Country/ No. breed	Microscopical positivity (OPG)	Genotyping at the gene loci	
						SSU, ACTIN	HSP70
Psittaciformes	**African grey parrot** (*Psittacus erithacus*)	1/0	NA	CZE/1	NA	NA	NA
		2/0	NA	CZE/2	NA	NA	NA
		2/0	NA	CZE/6	NA	NA	NA
		3/0	NA	CZE/7	NA	NA	NA
		4/0	NA	CZE/8	NA	NA	NA
		4/0	NA	CZE/9	NA	NA	NA
		4/0	NA	SVK/1	NA	NA	NA
		4/0	NA	SVK/2	NA	NA	NA
	Blue-and-yellow macaw (*Ara ararauna*)	2/0	NA	CZE/2	NA	NA	NA
		4/0	NA	CZE/8	NA	NA	NA
	Blue-fronted parrot (*Amazona aestiva*)	2/0	NA	CZE/1	NA	NA	NA
		4/0	NA	CZE/2	NA	NA	NA
		4/0	NA	CZE/3	NA	NA	NA
		1/0	NA	CZE/6	NA	NA	NA
		4/0	NA	CZE/7	NA	NA	NA
		5/0	NA	CZE/8	NA	NA	NA
		2/0	NA	CZE/9	NA	NA	NA
		3/0	NA	SVK/1	NA	NA	NA
		2/0	NA	SVK/2	NA	NA	NA
	Budgerigar (*Melopsittacus undulatus*)	8/1	28471	CZE/3	No	*C. avium*	*C. avium*
		9/1	26150	CZE/1	No	*C. baileyi*	*C. baileyi*
		7/1	35382	CZE/5	No	*C. baileyi*	*C. baileyi*
		7/0	NA	CZE/2	NA	NA	NA
		5/0	NA	CZE/4	NA	NA	NA
		6/0	NA	CZE/6	NA	NA	NA
		4/0	NA	CZE/7	NA	NA	NA
		8/0	NA	CZE/8	NA	NA	NA
		7/0	NA	CZE/9	NA	NA	NA
		10/0	NA	SVK/1	NA	NA	NA
		7/0	NA	SVK/2	NA	NA	NA
	Cockatiels (*Nymphicus hollandicus*)	18/3	34758	CZE/1	Yes (2,000)	*C. proventriculi*	*C. proventriculi*
			23772		No	*C. proventriculi*	*C. proventriculi*
			25137		Yes (2,000)	*C. baileyi*	*C. baileyi*
		6/1	35508	CZE/2	No	*C. proventriculi*	*C. proventriculi*

Table 2 (*Continued*)

Order	Host (scientific name)	Number of screened/ positive	ID of positive animal	Country/ No. breed	Microscopical positivity (OPG)	Genotyping at the gene loci	
						SSU, ACTIN	HSP70
		10/2	34306	CZE/5	No	*C. proventriculi*	*C. proventriculi*
			34320		Yes (30,000)	*C. proventriculi*	*C. proventriculi*
		6/2	34759	CZE/6	Yes (2,000)	*C. proventriculi*	*C. proventriculi*
			35506		Yes (16,000)	*C. proventriculi*	*C. proventriculi*
		14/3	14384	CZE/7	Yes (2,000)	*C. proventriculi*	*C. proventriculi*
			14385		Yes (6,000)	*C. proventriculi*	*C. proventriculi*
			17618		No	*C. baileyi*	*C. baileyi*
		25/4	34751	CZE/8	Yes (8,000)	*C. proventriculi*	*C. proventriculi*
			34331	CZE/8	Yes (6,000)	*C. proventriculi*	*C. proventriculi*
			34749	CZE/8	No	*C. proventriculi*	*C. proventriculi*
			34750	CZE/8	Yes (4,000)	*C. proventriculi*	*C. proventriculi*
		20/2	34305	SK/2	Yes (24,000)	*C. proventriculi*	*C. proventriculi*
			36598	SK/2	Yes (8,000)	*C. proventriculi*	*C. proventriculi*
		8/0	NA	CZE/3	NA	NA	NA
		5/0	NA	CZE/4	NA	NA	NA
		7/0	NA	CZE/9	NA	NA	NA
		5/0	NA	SK//1	NA	NA	NA
	Red-crowned parakeet (*Cyanoramphus novaezealandiae*)	4/1	19287	CZE/3	No	*C. avium*	*C. avium*
		2/0	NA	CZE/8	NA	NA	NA
		2/0	NA	CZE/9	NA	NA	NA
	Red-fronted parrot (*Poicephalus gulielmi*)	27/3	22127	CZE/8	No	*C. proventriculi*	*C. proventriculi*
			33626	CZE/8	No	*C. proventriculi*	*C. proventriculi*
			25108	CZE/8	No	*C. proventriculi*	*C. proventriculi*
	Red-rumped parrots (*Psephotus haematonotus*)	5/0	NA	CZE/3	NA	NA	NA
		6/0	NA	CZE/9	NA	NA	NA
	Rose-breasted cockatoo (*Eolophus roseicapilla*)	5/0	NA	CZE/8	NA	NA	NA
		2/0	NA	SK/2	NA	NA	NA
	Rose-ringed parakeet (*Psittacula krameri*)	4/0	NA	CZE/8	NA	NA	NA
		2/0	NA	CZE/9	NA	NA	NA

Table 2 (*Continued*)

Order	Host (scientific name)	Number of screened/ positive	ID of positive animal	Country/ No. breed	Microscopical positivity (OPG)	Genotyping at the gene loci	
						SSU, ACTIN	HSP70
	Rosy-faced lovebird (*Agapornis roseicollis*)	6/1	26156	CZE/1	No	*C. proventriculi*	*C. proventriculi*
		4/1	37339	CZE/7	Yes (4,000)	*C. proventriculi*	*C. proventriculi*
		6/1	37244	SK/2	No	*C. proventriculi*	*C. proventriculi*
		3/0	NA	CZE/3	NA	NA	NA
		1/0	NA	CZE/4	NA	NA	NA
		2/0	NA	CZE/6	NA	NA	NA
		3/0	NA	CZE/8	NA	NA	NA
		6/0	NA	SK/1	NA	NA	NA
	Senegal parrots (*Poicephalus senegalus*)	4/0	NA	CZE/1	NA	NA	NA
		3/0	NA	CZE/2	NA	NA	NA
		2/0	NA	CZE4	NA	NA	NA
		2/0	NA	CZE/6	NA	NA	NA
		2/0	NA	CZE/7	NA	NA	NA
		5/0	NA	CZE/8	NA	NA	NA
		4/0	NA	CZE/9	NA	NA	NA
		5/0	NA	SK1	NA	NA	NA
		3/0	NA	SK/2	NA	NA	NA
	White cockatoos (*Cacatua alba*)	5/0	NA	CZE/4	NA	NA	NA
		4/0	NA	CZE/8	NA	NA	NA
		3/0	NA	CZE/9	NA	NA	NA
		3/0	NA	SK/2	NA	NA	NA
	Yellow-collared lovebird (*Agapornis personatus*)	3/0	NA	CZE/7	NA	NA	NA
		5/0	NA	CZE/8	NA	NA	NA
Passeriformes	**Gouldian finch** (*Erythura gouldiae*)	10/1	22153	POL/1	No	*C. galli*	NA
		8/1	37242	POL/2	No	*C. baileyi*	*C. baileyi*
		5/0	NA	CZE/1	NA	NA	NA
		6/0	NA	CZE/5	NA	NA	NA
		7/0	NA	CZE/10	NA	NA	NA
	Island canary (*Serinus canaria*)	25/3	30887	CZE/10	No	avian genotype I	avian genotype I
			31040		No	avian genotype I	avian genotype I
			31056		No	avian genotype I	avian genotype I

Table 2 (*Continued*)

Order	Host (scientific name)	Number of screened/ positive	ID of positive animal	Country/ No. breed	Microscopical positivity (OPG)	Genotyping at the gene loci	
						SSU, ACTIN	HSP70
	Lesser goldfinch (*Carduelis psaltria*)	10/1	19900	POL/1	No	*C. galli*	NA
		5/1	24068	CZE/10	Yes (6,000)	*C. baileyi*	*C. baileyi*
		3/1	NA	CZE/1	NA	NA	NA
		5/0	NA	CZE8	NA	NA	NA
		3/0	NA	CZE/9	NA	NA	NA
	Zebra finch (*Taeniopygia guttata*)	3/1	19418	CZE/10	Yes (10,000)	*C. baileyi*	*C. baileyi*
		4/2	24067	POL/3	No	*C. galli*	NA
		4/0	37242	POL/3	No	*C. galli*	NA

cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) in 10 μl reactions.

Phylogenetic analyses

The nucleotide sequences of each gene obtained in this study were edited using the ChromasPro 2.4.1software (Technelysium, Pty, Ltd., South Brisbane, Australia) and aligned with each other and with reference sequences from GenBank using MAFFT version 7 online server with automatic selection of alignment mode (http://mafft.cbrc.jp/alignment/software/). Phylogenetic analyses were performed and best DNA/Protein phylogeny models were selected using the MEGA7 software (Guindon and Gascuel 2003; Tamura et al. 2011). Phylogenetic trees were inferred by the maximum likelihood (ML) method, with the substitution model that best fit the alignment selected using the Bayesian information criterion. The Tamura 3-parameter model (Tamura 1992) was selected for SSU and HSP70 alignments, and the general time reversible model (Tavaré 1986) was selected for actin alignment. Bootstrap support for branching was based on 1000 replications. Phylograms were drawn using the MEGA7 and were manually adjusted using CorelDrawX7. Sequences of SSU, actin and HSP70 derived in this study have been deposited in GenBank under accession numbers MK311133–MK311180.

Transmission studies

Animals

Groups of adult cockatiels, adult budgerigars (*Melopsittacus undulates*), one-days-old and 21-day-old chickens (*Gallus gallus* f. *domestica*), and eight-week-old SCID mice (*Mus mucus*; strain C.B-17), each consisting of five animals, were used for experimental infection studies. In addition, three animals from each host species were used as negative control. Three weeks prior to experimental infections, animals were screened every other day for the presence of specific DNA and oocysts of *Cryptosporidium* spp., except chickens, which were hatched under monitored conditions in the laboratory. Cockatiels and budgerigars originated from breeders with *Cryptosporidium*-free birds (Czech Republic) and SCID mice from Charles River (Germany).

Animal care

To prevent environmental contamination with oocysts, laboratory rodents were housed in plastic cages (TOP-VELAZ, Prague, Czech Republic). Chickens were housed in the plastic boxes that were appropriately sized for their age. An external source of heat was used in the first five days. Budgerigars and cockatiels were kept in bird cages, appropriate to animal species. All animals were supplied with a sterilized diet and sterilized water *ad libitum*.

Animal caretakers wore new disposable coveralls, shoe covers, and gloves every time they entered the experimental room. All wood-chip bedding, faeces, and disposable protective clothing were sealed in plastic bags, removed from the experimental room and incinerated. All housing, feeding and experimental procedures were conducted under protocols

approved by the Institute of Parasitology, Biology Centre and Central Commission for Animal Welfare, Czech Republic (Protocols No. 115/2013 and 35/2018).

Experimental design

Each animal was inoculated orally by stomach tube with 10,000 purified viable oocysts suspended in 200 μl of distilled water. Animals serving as negative controls were inoculated orally by stomach tube with 200 μl of distilled water. Faecal samples from all animals were screened daily

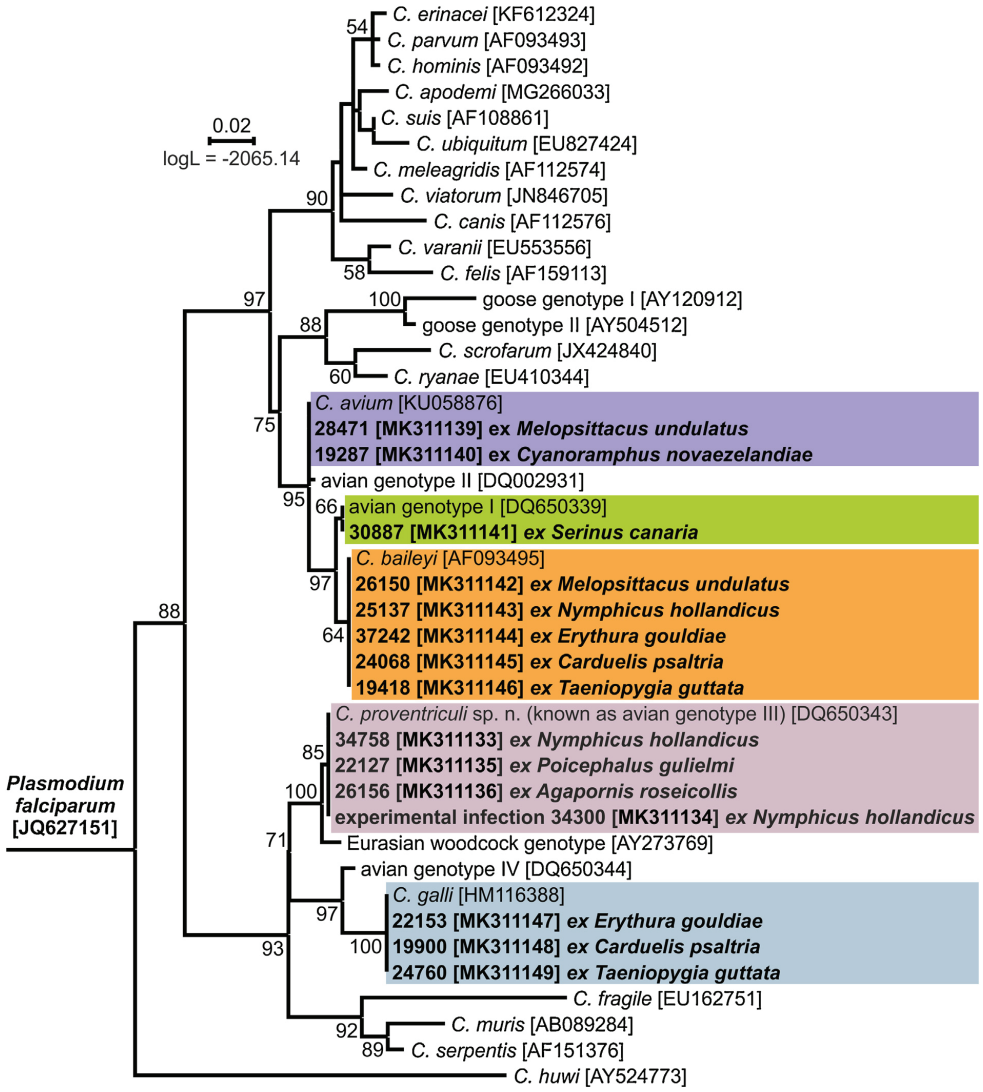

Fig. 1. Maximum likelihood tree based on partial small subunit ribosomal RNA gene sequences of *Cryptosporidium*, including sequences obtained in this study (bolded). The alignment contained 458 base positions in the final dataset. Numbers at the nodes represent the bootstrap values with more than 50% bootstrap support from 1000 pseudoreplicates. The branch length scale bar, indicating the number of substitutions per site, is given in the tree. Sequences from this study are identified by isolate number (e.g. 22127).

for the presence of *Cryptosporidium* oocyst using ACMV staining and the presence of *Cryptosporidium* specific DNA was confirmed using nested PCR targeting the SSU gene. All experiments were terminated 30 days post infection (DPI). Infection intensity was reported as the number of oocysts per gram (OPG) of faeces, as previously described by Kváč et al. (2007). In addition, faecal consistency and colour and general health status were examined daily.

Histopathological and scanning electron microscopy examinations

An animal from each host group that was positive for *Cryptosporidium proventriculi* sp. n. was euthanized at 20 DPI (this time was selected based on preliminary results; data not shown). The oesophagus, ventriculus, proventriculus and entire small and large intestine was divided into 1 cm sections

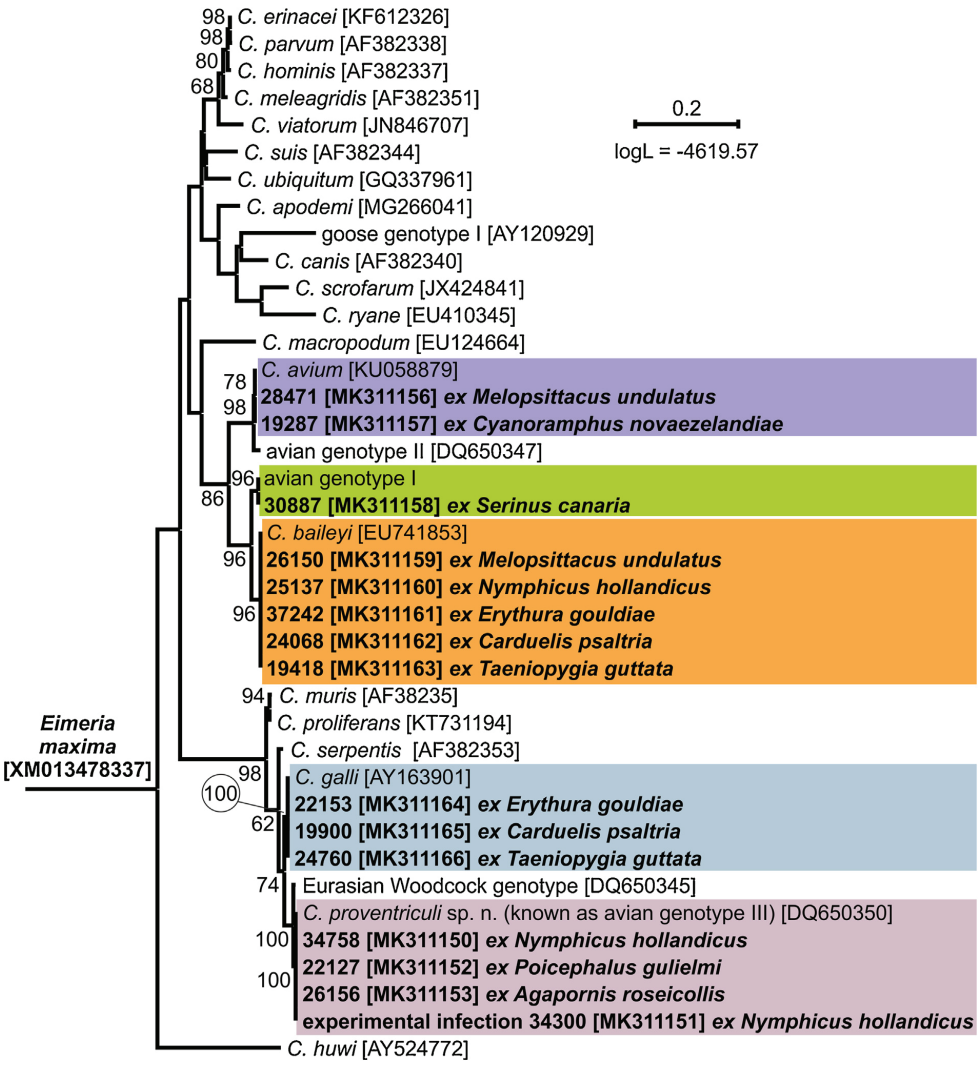

Fig. 2. Maximum likelihood tree based on partial sequences of gene coding actin of *Cryptosporidium*, including sequences obtained in this study (highlighted and bolded). The alignment contained 969 base positions in the final dataset. Numbers at the nodes represent the bootstrap values with more than 50% bootstrap support from 1000 pseudoreplicates. The branch length scale bar, indicating the number of substitutions per site, is given in the tree. Sequences from this study are identified by isolate number (e.g. 22127).

and samples were processed for histology, scanning electron microscopy (SEM) and DNA sequencing. Specimens for histology were fixed in 4% buffered formalin and processed by the standard paraffin method. Sections (5 μm) for histology were stained with haematoxylin and eosin, Wolbach's modified Giemsa stain and periodic acid–Schiff stains. Specimens for SEM were fixed overnight at 4 °C in 2.5% glutaraldehyde in 0.1 M phosphate buffer, washed three times for 15 min in the same buffer, post-fixed in 2% osmium tetroxide in 0.1 M phosphate buffer for 2 h at room temperature and finally washed three times for 15 min in the same buffer. After dehydration in a graded acetone series, specimens were dried using the critical point technique, coated with gold and examined using a JEOL JSM-7401F-FE SEM.

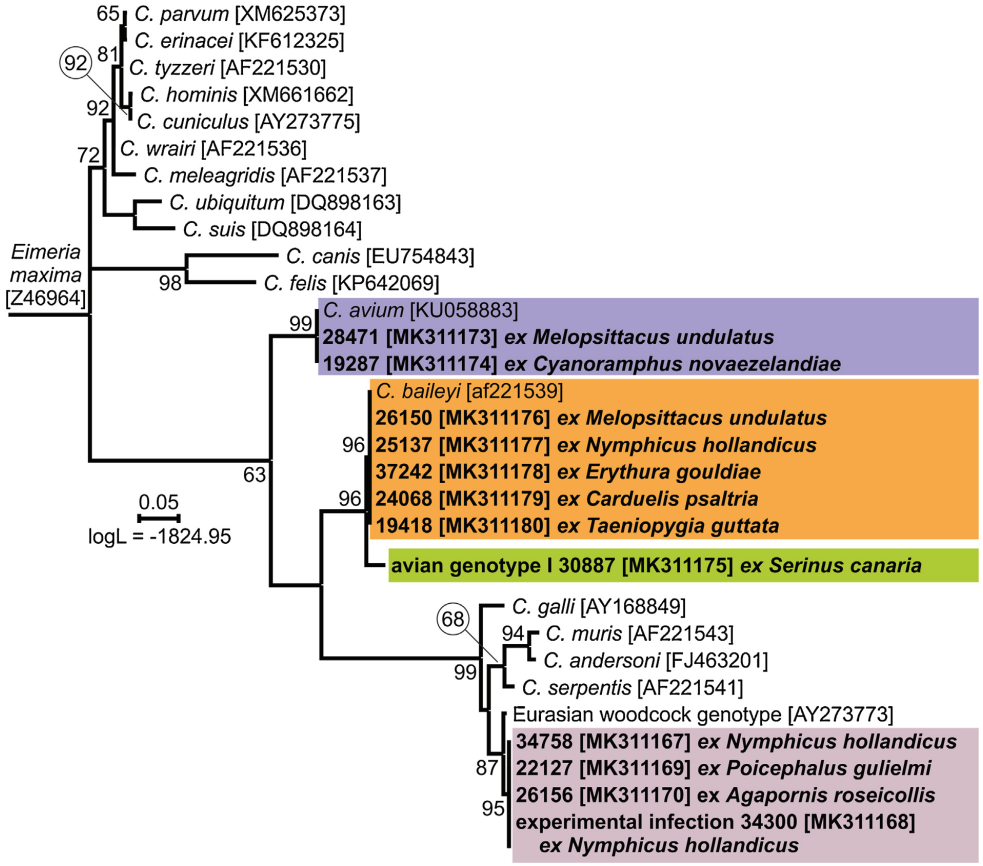

Fig. 3. Maximum likelihood tree based on partial sequences of gene coding 70 kDa Heat Shock Protein of *Cryptosporidium*, including sequences obtained in this study (highlighted and bolded). The alignment contained 488 base positions in the final dataset. Numbers at the nodes represent the bootstrap values with more than 50% bootstrap support from 1000 pseudoreplicates. The branch length scale bar, indicating the number of substitutions per site, is given in the tree. Sequences from this study are identified by isolate number (e.g. 22127).

Statistical analysis

The hypothesis tested in the analysis of oocyst morphometry was that two-dimensional mean vectors of measurement are the same in the two populations being compared. Hotelling's T2 test was used to test the null hypothesis. Analyses were performed using the program R 3.5.0. (https://www.r-project.org/).

Results

Out of 500 faecal samples from Psittaciformes and Passeriformes birds, 37 (7.4%) were positive for the presence of specific DNA of *Cryptosporidium* spp. and 15 (3.0%) were microscopically positive for the presence of oocysts of *Cryptosporidium* sp. (Table 2). All microscopically positive samples were also positive using PCR.

Out of 37 birds positive for *Cryptosporidium*, 37, 37 and 33 were genotyped by sequence analysis of SSU, actin and HSP70 genes, respectively (Table 2). The remaining four positive samples failed to amplify at the HSP70 locus. Sequence analysis revealed the presence of five different *Cryptosporidium* spp., clustering with *C. avium*, *C. baileyi*, *C. galli*, *Cryptosporidium* avian genotype I and *Cryptosporidium* avian genotype III in ML trees inferred from sequences of SSU, actin and HSP70 (Figs. 1–3). *Cryptosporidium* avian genotype III is described here as a new species, *Cryptosporidium proventriculi* sp. n., and this name will be used in the following text.

Cryptosporidium avian genotype I (n = 3) and *C. galli* (n = 4) were found only in Passeriformes birds, and infected birds did not shed microscopically detectable oocysts

(Table 2). *Cryptosporidium baileyi* (n = 7) was detected in species from the orders Psittaciformes and Passeriformes, with infection intensities less than 10,000 OPG. *Cryptosporidium avium* was detected in two species from the order Psittaciformes. *Cryptosporidium proventriculi* sp. n., which was also exclusive to Psittaciformes, was detected in 21 birds from three species (Table 2), and was the most abundant species detected in this study. Out of 21 birds positive for *C. proventriculi* sp. n., 12 (57%) shed microscopically detectable oocysts, with an infection intensity ranging from 2,000 to 30,000 OPG. Naturally infected cockatiels (ID Nos. 34305, 34320, 34751 and 35506), which were the source of oocysts used in experimental infections, shed oocysts for more than five months (data not shown). None of the monitored birds had diarrhoea at the time of the screening.

Cryptosporidium proventriculi sp. n.

Fifteen cockatiels (12.1%), three red-fronted parrots (11.0%) and three rosy-faced lovebirds (9.7%) were positive for the presence of *C. proventriculi* sp. n. DNA, of which 12 cocktails and one rosy-faced lovebird shed oocysts detectable by microscopy, with infection intensity ranging from 2,000 to 30,000 OPG (Table 2). Oocyst used for experimental infections had >90% viability, determined by PI staining. SCID mice, budgerigars and chickens were not susceptible to infection with *C. proventriculi* sp. n. oocysts, as determined by PCR and microscopic examination of faecal samples and histological and molecular examination of gastrointestinal tract tissue.

Oocysts of *C. proventriculi* sp. n. were infectious for all cockatiels, with oocysts and specific DNA first detected at six DPI (Fig. 4). Following first detection, specific DNA of *C. proventriculi* sp. n. was detected in the faeces of all animals for the duration of the experiment. In contrast, oocysts were detected by microscopy almost every day (Fig. 4). The infection intensity of *C. proventriculi* sp. n. in cockatiels ranged from 4,000 to 60,000 OPG. Infected birds showed no symptoms of disease and a cockatiel necropsied at 20 DPI had no macroscopic signs of cryptosporidiosis. Developmental stages of *C. proventriculi* sp. n. were not detected by histology, but scanning electron microscopy showed the presence of developmental stages attached to the microvilli in the proventriculus and ventriculus (Fig. 5). Pathological changes were not observed.

At the SSU locus, all isolates of *C. proventriculi* sp. n., from naturally and experimentally infected birds, shared 100% identity with each other and with *Cryptosporidium* avian genotype III from different Psittaciformes hosts worldwide (e.g. HM116385, KX668210 or HM116386; see Table 1 for more details). At the actin locus, all *C. proventriculi* sp. n. isolates shared 100% identity with each other and with GenBank sequences of *Cryptosporidium* avian genotype III obtained from peach-faced lovebirds and cockatiels (e.g. AB471655–AB471658, AB471659 or DQ650350). Partial sequences of the HSP70 locus shared 100% identity with each other and shared 98.7% identity with *Cryptosporidium* Eurasian woodcock genotype (AY273773), which was the most similar sequence reported in GenBank.

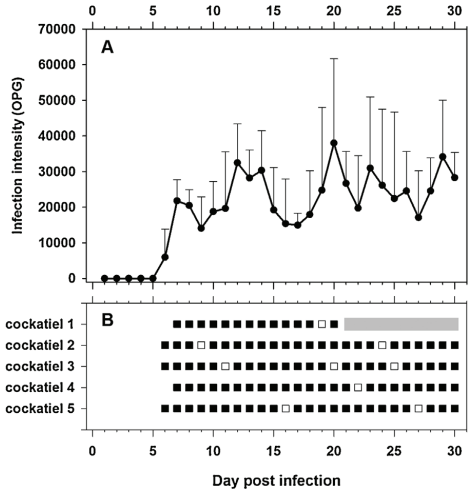

Fig. 4. Course of infection of *Cryptosporidium proventriculi* sp. n. in experimentally infected cockatiels (*Nymphicus hollandicus*). **A)** Infection intensity as number of oocysts per gram of the faeces (OPG) and **B)** daily shedding of *C. proventriculi* based on coprological and molecular examination of faces; any square indicates detection of specific DNA, black square indicates microscopic detection of oocysts and grey rectangle indicates sacrifice and dissection.

Taxonomic summary

Description: Oocysts are shed fully sporulated with four sporozoites and an oocyst residuum.

Oocysts of *C. proventriculi* n. sp. originated from naturally infected cockatiels measured 6.70–8.40 μm (mean ± S.D. = 7.35 ± 0.41 μm) × 5.10–6.3 μm (mean ± S.D. = 5.70 ± 0.32 μm) with a length/width ratio of 1.08–1.41 (mean ± S.D. = 1.23 ± 0.11) and were morphometrically identical to those recovered from experimentally infected cockatiels measured 6.60–8.40 μm (mean ± S.D. = 7.37 ± 0.44 μm) × 5.00–6.40 μm (mean ± S.D. = 5.80 ± 0.35 μm) with a length/width ratio of 1.06–1.43 (mean ± S.D. = 1.25 ± 0.10) (Fig. 6). Morphology and morphometry of other developmental stages are unknown.

Type host: cockatiel (*Nymphicus hollandicus*)

Other natural hosts: barred parakeet (*Bolborhynchus lineola*), blue-fronted parrot (*Amazona aestiva*), *Forpus* sp., galah (*Eolophus roseicapilla*), green-winged saltator (*Serinus canaria*), lovebird (*Agapornis* sp.), java sparrow (*Padda oryzivora*), peach-faced lovebird (*Agapornis roseicollis*), red-billed blue magpie (*Urocissa erythrorhyncha*), red-billed toucan (*Ramphastos tucanus*), red-fronted par-

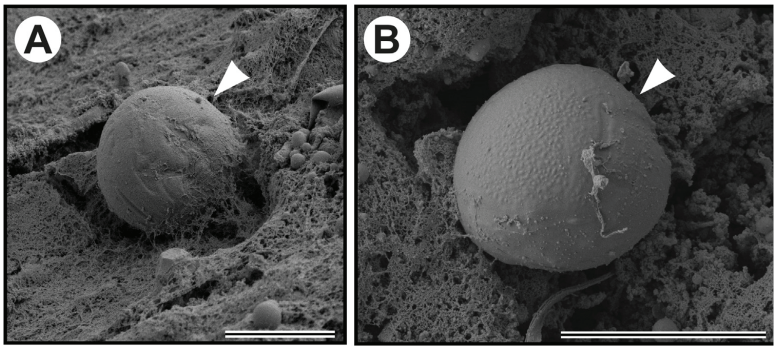

Fig. 5. Scanning electron photomicrograph of the **A)** proventriculus and **B)** ventriculus of a cockatiel (*Nymphicus hollandicus*). Attached developmental stage of *Cryptosporidium proventriculi* sp. n. (arrowhead). Bar = 10 μm.

rot (*Poicephalus gulielmi*), rosy-faced lovebird (*Agapornis roseicollis*), rufous-collared sparrow (*Zonotrichia capensis*), saffron toucanet (*Pteroglossus bailloni*), seagul (*Chroicocephalus brunnicephalus* and *ridibundus*), *Sporophila* sp., sun parakeet (*Aratinga solstitialis*) and toco toucan (*Ramphastos toco*) (see Tables 1 and 2).

Type locality: České Budějovice (Czech Republic)

Other localities: Czech Republic (České Budějovice 48°58′29″ N, 14°28′29″ E; Lanžhot 48°43′28.03″ N, 16°58′0.97″ E; Osek u Plzně 49°26′36.33″ N, 14°18′0.43″ E; Strakonice 49°15′41.39″ N, 13°54′8.63″ E and Tvrdonice 48°45′37.81″ N, 16°59′40.03″ E), Slovakia (Košice 48°42′0″ N, 21°15′0″ E and Žilina 49°13′22″ N, 18°44′24″ E), Poland (Wroclaw 51°7′0″ N, 17°2′0″ E).

Site of infection: proventriculus and ventriculus (Fig. 5)

Distribution: Australia, Brazil, China, India, Japan, Poland, Slovakia, Spain, Thailand and USA

Prepatent period: 6 DPI (cockatiel)

Patent period: at least 30 DPI in experimentally infected cockatiels and more than 5 months in naturally infected cockatiels.

Type material/hapanotype: Tissue samples in 10% formaldehyde and histological sections of infected ventriculus (nos. 176/2017 and 177/2017) and provetriculus (nos. 199/2017); genomic DNA isolated from faecal samples of naturally (isolation no. 34320) and experimentally (isolation no. 34300) infected cockatiel; genomic DNA isolated from proventriculus and ventricular and of experimentally infected cockatiel (isolation nos. 34300); digital photomicrographs nos. DIC 1–13/34300, MV 1–11/34300, IF 1–9/34300, HI 176–177, 199/2017 and SEM 199/2017) and faecal smear slides with oocysts stained by ACMV staining from experimentally infected cockatiel (nos. 10/34300, 11/34300 and 12/34300). Specimens deposited at the Institute of Parasitology, Biology Centre of the Czech Academy of Sciences, Czech Republic.

Reference sequences: Partial sequences of SSU, actin and HSP70 genes were deposited at GenBank under Acc. Nos. MK311133, MK311150 and MK311167, respectively.

Etymology: The species name is derived from the Latin noun ‘proventriculus’, because the described *Cryptosporidium* species predominantly inhabits this part of digestive tract of its hosts.

Differential diagnosis: Oocysts in faecal smears showed typical *Cryptosporidium* ACMV (Fig. 6) and Ziehl-Neelsen (Fig. 6) staining characteristics. Fixed *C. proventriculi* sp. n. oocysts are detectable with a FITC conjugated anti-*Cryptosporidium* oocyst wall antibody developed primarily for *C. parvum* (Fig. 6). Oocysts of *C. proventriculi* are larger than those of *C. avium* and *C. baileyi* (P = 0.001) and marginally smaller than *C. galli*. *Cryptosporidium proventriculi* sp. n. can be differentiated genetically from other *Cryptosporidium* spp. based on sequences of SSU, actin and HSP70 genes.

Discussion

Birds are naturally infected with several *Cryptosporidium* spp. While *C. meleagridis*, *C. galli* and *C. baileyi* infect a large number of birds across several avian orders, *C. avium* and most of the avian genotypes exhibit a narrow host specificity (Holubová et al. 2016). Although *C. proventriculi* sp. n. has been reported from birds in the orders Psittaciformes, Passeriformes, Piciformes and Anseriformes, it has been reported predominantly in Psittaciformes birds (Cano et al. 2016; Ferrari et al. 2018; Koompapong et al. 2014; Li et al. 2015; Ravich et al. 2014). Consistent with those reports, we found *C. proventriculi* sp. n. exclusively in Psittaciformes birds, and we identified the red-fronted parrot and rosy-faced lovebird as novel hosts in that order.

Cryptosporidiosis in birds manifests in various clinical forms depending on the species of *Cryptosporidium* and the site of infection (Nakamura and Meireles 2015). Despite the relatively large number of studies and descriptions of sev-

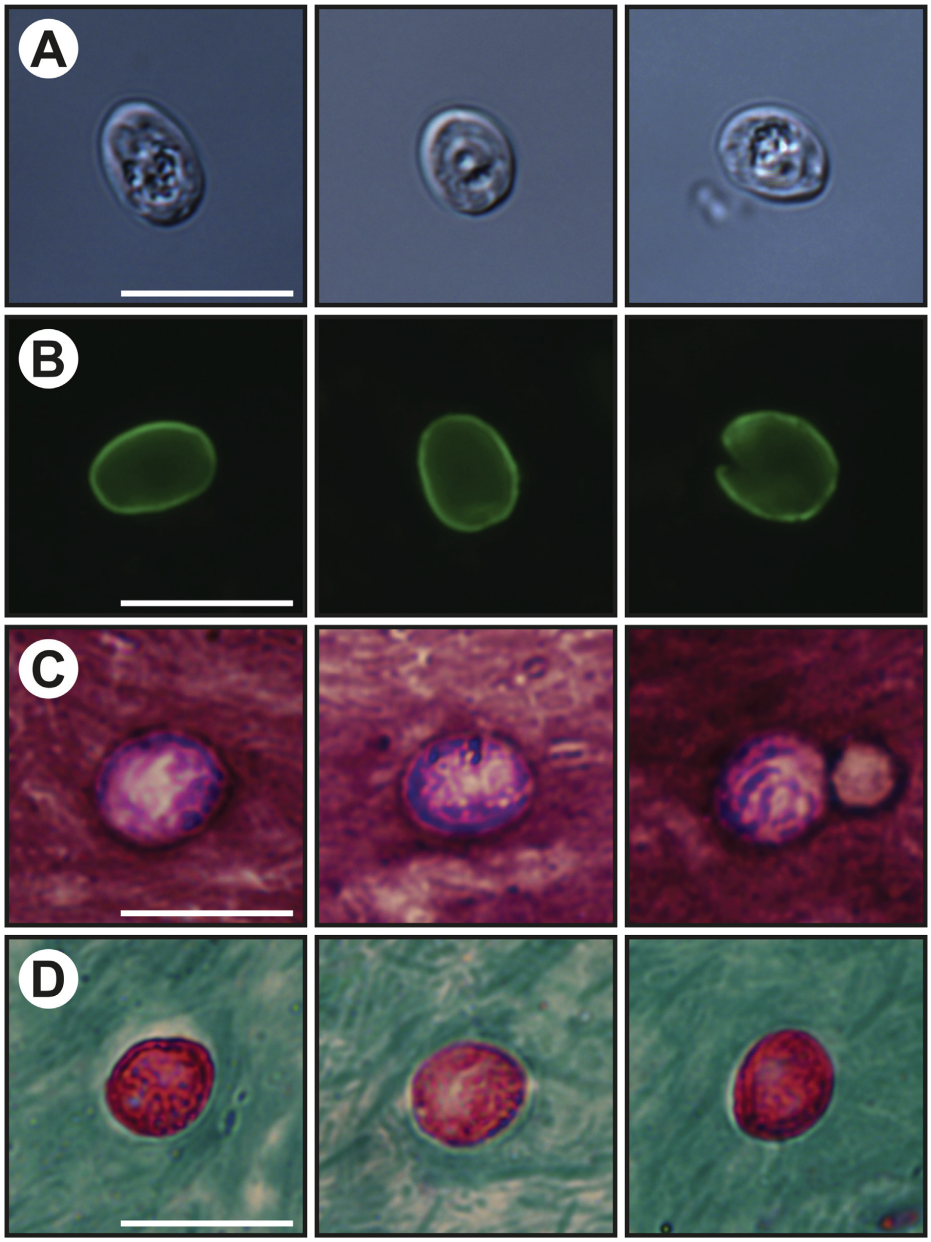

Fig. 6. *Cryptosporidium proventriculi* sp. n. oocysts visualized in various preparations: **A)** differential interference contrast microscopy, **B)** labelling with anti-*Cryptosporidium* FITC-conjugated antibody, **C)** aniline-carbol-methyl violet staining and **D)** Ziehl-Neelsen staining. Bar = 10 μm.

eral *Cryptosporidium* genotypes, knowledge of the course of infection and disease presentation of *Cryptosporidium* in birds remains quite poor. *Cryptosporidium galli* is the only well-studied gastric *Cryptosporidium* species in birds. Unlike gastric *Cryptosporidium* species infecting mammals, which generally do not cause morbidity and mortality, *C. galli* is associated with clinical disease and high mortality in birds (Blagburn et al. 1987; Blagburn et al. 1990; Morgan et al. 2001; Pavlásek 1999, 2001). However, similar to Ng et al. (2006), we found that *C. proventriculi* does not cause clinical disease or mortality in naturally or experimentally infected birds. Ravich et al. (2014) reported proventricular and ventricular cryptosporidiosis in 31 birds at necropsy and found evidence that *Cryptosporidium* was the likely cause of death in 10 of those birds. The authors identified *C. proventriculi* from samples of two birds, which were the only samples subjected to genotyping, but it is not clear whether those birds were among the 10 that likely died from cryptosporidiosis. While *C. proventriculi* could be the cause of death in those birds, infection by other *Cryptosporidium* species cannot be ruled out. Makino et al. (2010) described clinical gastrointestinal signs such as chronic vomiting, melena and weight loss in 20 out of 37 peach-faced lovebirds naturally infected with *C. proventriculi*. Radiographic examination showed enlargement of the isthmi and thickened proventricular walls in 16 of the 20 symptomatic birds. Necropsy and histopathologic examination of three dead birds showed enlargement of the proventriculus and isthmi with mucosal hypertrophy observed on the sagittal plane. The progressive weight loss with severely atrophied thoracic muscles was characteristic for all three affected birds. Histopathologically, extensive hyperplasia of the ductal epithelium of the proventricular glands was observed. Similarly, Blagburn et al. (1990) and Morgan et al. (2001) reported histopathological changes similar to those caused by *C. galli* infection in the Australian diamond firetail finch and other finches infected with *Cryptosporidium* sp. and *C. proventriculi*, respectively. In contrast, we found no histopathological changes in birds infected with *C. proventriculi*. These differences can be explained by the length of the ongoing infection. While our experiments lasted only 30 days, naturally infected birds could be infected for many months. Similarly, histopathological changes and clinical signs were observed in mammals chronically infected with the gastric species *C. proliferans*, *C. andresoni* or *C. muris* (Anderson 1987; Esteban and Anderson 1995; Kváč et al. 2016; Ozkul and Aydin 1994; Pospischil et al. 1987).

Course of infection, including prepatent and patent period, of avian-derived *Cryptosporidium* is currently known only in the four valid avian species (Current et al. 1986; Holubová et al. 2016; Ryan et al. 2003b; Slavin 1955). The prepatent period of *C. proventriculi* sp. n. (6 DPI) was similar to *C. meleagridis* and *C. baileyi*, which infect the intestine (4–8 days; Hornok et al. 1999; Lindsay et al. 1988; Rhee et al. 1991; Tůmová et al. 2002), and much shorter than the 25 days reported for *C. galli* infection in the proventriculus of chickens (Pavlásek 2001).

Oocysts of *C. proventriculi* sp. n. are morphometrically identical to those reported as *Cryptosporidium* avian genotype III (Ng et al. 2006). Although they are smaller than those of *C. galli* (Ryan et al. 2003b) and Eurasian woodcock genotype (Ryan et al. 2003a), the difference is marginal and not practically useful for differentiation (Horčičková et al. 2018).

Cryptosporidium proventriculi sp. n. is genetically distinct from other known species of *Cryptosporidium*, sharing 98.4 and 94.5% sequence identity, respectively, with *Cryptosporidium* Eurasian woodcock genotype and *C. galli* at the SSU locus and sharing 98.9 and 96.9% identity, respectively, at the actin locus. In comparison, *C. muris* and *C. andersoni* share 96–99% identity and *C. hominis* and *C. parvum* share 98–99% identity at these loci.

In conclusion, morphological, genetic, and biological data support the establishment of *Cryptosporidium* avian genotype III as a new species. According to ICZN and criteria for naming species, we propose the name *Cryptosporidium proventriculi*.

Author contributions

N.H., M.K. and B.S. conceptualised the project; Z.K., D.R., N.H. and V.Z. collected samples; N.H., M.K., V.Z. and Z.L. carried out the research; M.K., N.H. and J.M. performed phylogenetic analysis; B.S. and R.K. performed histology and electron microscopy analysis; L.H. took care of experimental animals and M.K., N.H., J.M. wrote the manuscript. All authors read and approved the final manuscript.

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7.6 Příloha VI

Description of *Cryptosporidium ornithophilus* n. sp. (Apicomplexa: Cryptosporidiidae) in farmed ostriches.

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Parasites & Vectors

RESEARCH Open Access

Description of *Cryptosporidium ornithophilus* n. sp. (Apicomplexa: Cryptosporidiidae) in farmed ostriches

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Abstract

Background: Avian cryptosporidiosis is a common parasitic disease that is caused by five species, which are well characterised at the molecular and biological level, and more than 18 genotypes for which we have limited information. In this study, we determined the occurrence and molecular characteristics of *Cryptosporidium* spp. in farmed ostriches in the Czech Republic.

Methods: The occurrence and genetic identity of *Cryptosporidium* spp. were analysed by microscopy and PCR/sequencing of the small subunit rRNA, *actin*, *HSP70* and *gp60* genes. *Cryptosporidium* avian genotype II was examined from naturally and experimentally infected hosts and measured using differential interference contrast. The localisation of the life-cycle stages was studied by electron microscopy and histologically. Infectivity of *Cryptosporidium* avian genotype II for cockatiels (*Nymphicus hollandicus* (Kerr)), chickens (*Gallus gallus* f. *domestica* (L.)), geese (*Anser anser* f. *domestica* (L.)), SCID and BALB/c mice (*Mus musculus* L.) was verified.

Results: A total of 204 individual faecal samples were examined for *Cryptosporidium* spp. using differential staining and PCR/sequencing. Phylogenetic analysis of small subunit rRNA, *actin*, *HSP70* and *gp60* gene sequences showed the presence of *Cryptosporidium* avian genotype II ($n=7$) and *C. ubiquitum* Fayer, Santín & Macarisin, 2010 IXa ($n=5$). Only ostriches infected with *Cryptosporidium* avian genotype II shed oocysts that were detectable by microscopy. Oocysts were purified from a pooled sample of four birds, characterised morphometrically and used in experimental infections to determine biological characteristics. Oocysts of *Cryptosporidium* avian genotype II measure on average 6.13 × 5.15 µm, and are indistinguishable by size from *C. baileyi* Current, Upton & Haynes, 1986 and *C. avium* Holubová, Sak, Horčičková, Hlásková, Květoňová, Menchaca, McEvoy & Kváč, 2016. *Cryptosporidium* avian genotype II was experimentally infectious for geese, chickens and cockatiels, with a prepatent period of four, seven and eight days post-infection, respectively. The infection intensity ranged from 1000 to 16,000 oocysts per gram. None of the naturally or experimentally infected birds developed clinical signs in the present study.

Conclusions: The molecular and biological characteristics of *Cryptosporidium* avian genotype II, described here, support the establishment of a new species, *Cryptosporidium ornithophilus* n. sp.

Keywords: *Cryptosporidium* avian genotype II, *Cryptosporidium ornithophilus* n. sp., *C. ubiquitum*, Occurrence, Oocyst size, PCR, Experimental infections

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Background

The genus *Cryptosporidium* Tyzzer, 1910 comprises protist parasites that infect epithelial cells in the microvillus border, primarily of the gastrointestinal tract, of all classes of vertebrates [1]. Until recently, only three bird-derived *Cryptosporidium* species, *C. baileyi* Current, Upton & Haynes, 1986, *C. galli* Pavlásek, 1999 and *C. meleagridis* Slavin, 1955, were described in birds [2–4]. Even with the recent descriptions of *C. avium* Holubová, Sak, Horčičková, Hlásková, Květoňová, Menchaca, McEvoy & Kváč, 2016 [5] and *C. proventriculi* Holubová, Zikmundová, Limpouchová, Sak, Konečný, Hlásková, Rajský, Kopacz, McEvoy & Kváč, 2019 [6], the number of described species in birds remains low relative to that in mammals. Eighteen *Cryptosporidium* genotypes (*Cryptosporidium* sp. YS-2017 genotype, avian genotype I, avian genotype IV, avian genotypes VI-IX, black duck genotype, Euro-Asian woodcock genotype, duck genotype, goose genotypes I-IV and goose genotype Id and finch genotypes I-III) have been identified [7–15], primarily based on small subunit rRNA sequence data, across 17 avian orders worldwide [8, 9, 13, 16, 17]. Although avian *Cryptosporidium* spp. have been studied more frequently in recent years, research has been biased towards *Cryptosporidium* in poultry and pet birds, with comparatively little attention paid to *Cryptosporidium* in other bird groups [16, 18].

Unlike *C. baileyi*, which infects a broad range of birds from different orders, many recently described *Cryptosporidium* species and genotypes appear to have a relatively narrow host range. For example, *Cryptosporidium* avian genotype VI appears to be restricted to North American red-winged blackbirds [8], and *Cryptosporidium* goose and duck genotypes have been found only in anseriform birds [11, 15]. Similarly, *C. avium* and *Cryptosporidium* avian genotype I are almost exclusively found in psittacines and passerines, respectively [5–7, 19]. *Cryptosporidium* avian genotype II has been found predominantly in ostriches but also in other species within the order Struthioniformes as well as orders Galliformes and Psittaciformes (Table 1).

Cryptosporidium in ostriches was first reported in 1993 [20] and there have been several reports since then, although most have not described the molecular characteristics of isolates [20–31]. Where molecular studies have been performed, with the exception of the rodent-specific *C. muris* Tyzzer, 1907, which was detected in 22 birds [32], *C. baileyi* [4, 32–35] and *Cryptosporidium* avian genotype II [19, 36] have been the only *Cryptosporidium* spp. reported in ostriches. While the biology of *C. baileyi* is well studied, there is limited information about *Cryptosporidium* avian genotype II.

In the present study, we report on the occurrence of *Cryptosporidium* spp. in farmed ostriches. For the most prevalent genotype in ostriches, *Cryptosporidium* avian genotype II, we further describe oocyst morphometry, experimental host specificity, developmental stage localization and molecular characteristics. Based on the collective data from this and previous studies, we conclude that *Cryptosporidium* avian genotype II is genetically and biologically distinct from the species of *Cryptosporidium* considered valid, and propose the name *Cryptosporidium ornithophilus* n. sp. for this genotype.

Methods

Specimens studied

Faecal samples were collected from ostriches on four farms in the Czech Republic. Faecal samples from juvenile (aged 9–12 months) and adult (older than three years) ostriches were individually collected into sterile plastic vials and stored at 4–8 °C until subsequent processing. Faecal smears were prepared from each sample, stained with aniline-carbol-methyl violet (ACMV), and examined for the presence of *Cryptosporidium* spp. oocysts [37]. Faecal samples were also screened for the presence of *Cryptosporidium*-specific DNA by PCR/sequencing (described below). Oocysts of *C. ornithophilus* n. sp. were purified from pooled faecal samples from a naturally infected juvenile common ostrich (no. 43588, *Struthio camelus* L.) kept on the farm number 4 using caesium chloride gradient centrifugation [38]. Purified oocysts were used for morphometry and preparation of the inoculum. The propidium iodide (PI) staining was used for test of oocysts viability [39]. *Cryptosporidium ornithophilus* n. sp. oocysts from a common ostrich were pooled and used to infect a single one-day-old chickens (chicken 0; *Gallus gallus* f. *domestica*). Oocysts recovered from the faeces of chicken 0 were used to infect other experimental animals. The purity of *C. ornithophilus* n. sp. isolate before performing the experimental infection and taking the measurements, and during the experiments was verified by the following procedure. The sequence of the original isolate (ostrich) was compared to the sequence obtained from chicken 0 and from tissue specimens and faecal samples of experimentally inoculated animals (below). The oocyst size of the original isolate was compared with isolates obtained from susceptible hosts.

Oocyst morphometry

Oocysts of *C. ornithophilus* n. sp. from naturally and experimentally infected hosts (50 oocysts from each isolate) were examined and length and width measurements were taken using differential interference contrast (DIC) at 1000× magnification. All measurements

Table 1 The occurrence of *Cryptosporidium* avian genotype II in birds from the orders Galliformes, Psittaciformes and Struthioniformes demonstrated on the basis of molecular tools amplifying partial sequences of *Cryptosporidium* small subunit ribosomal RNA (*SSU*), *actin* and 70 kDa heat-shock protein (*HSP70*) genes

Host	Country	Locus (GenBank ID)	No. positive/no. screened	Reference
Chicken (*Gallus gallus*)[a]	China	*SSU* (JX548291-92)	6/385	[57]
Ostrich (*Struthio camelus*)[c]	Vietnam	*SSU* (AB696811)	110/464	[36]
	Brazil	*SSU* (DQ002931) *Actin* (DQ002930) *HSP70* (DQ002929)	1/1 1/1 1/1	[59]
	Brazil	*SSU* (DQ650341)[d] *Actin* (DQ650348)[d]	6/41 6/41	[19]
Cockatiel (*Nymphicus hollandicus*)[b]	Australia	*SSU* (DQ002931)[d] *Actin* (DQ002930)[d]	3/ns 2/ns	[7]
Eclectus (*Eclectus roratus*)[b]	Australia	*SSU* (DQ650340) *Actin* (DQ650347)	2/ns 1/ns	[7]
Galah (*Eolophus roseicapilla*)	Australia	*SSU* (DQ650341) *Actin* (DQ650348)	1/ns 1/ns	[7]
Major Mitchell cockatoo (*Cacatua leadbeateri*)[b]	Australia	*SSU* (DQ002931)[d] *Actin* (DQ002930)[d]	3/ns 1/ns	[7]
Alexandrine (*Psittacula eupatria*)[b]	Australia	*SSU* (DQ002931)[d]	1/ns	[7]
Princess parrot (*Polytelis alexandrae*)[b]	Australia	*SSU* (DQ002931)[d]	1/ns	[7]
Sun conure (*Aratinga solstitialis*)[b]	Australia	*SSU* (DQ002931)[d]	1/ns	[7]
White-eyed parakeet (*Aratinga leucophthalma*)[b]	Brazil	*SSU* (DQ650341)[d]	1/ns	[56]

[a] Galliformes

[b] Psittaciformes

[c] Struthioniformes

[d] The sequence obtained in the present study has not been stored in the GenBank database and was identical to sequence published previously

Abbreviation: ns, not specified

are in micrometres and are given as the range followed by the mean ± standard deviation (SD) in parentheses. These measurements were used to calculate the length-to-width ratio. Sample containing purified *C. parvum* Tyzzer, 1912 oocysts from a naturally infected Holstein calf was used as a size control ($n = 50$). Size of oocysts was measured using the same microscope and by the same person. Each slide was screened a meandering path to prevent repeated measurement of an oocyst. Additionally, different staining methods were used for visualisation of oocysts. Faecal smears with *C. ornithophilus* n. sp. and *C. parvum* (data not shown) oocysts were stained by ACMV, modified Ziehl-Neelsen [ZN; 40], phenol staining [AP; 41] and labelled with genus-specific FITC-conjugated antibodies (IFA; *Cryptosporidium* IF Test, Crypto cel, Cellabs Pty Ltd., Brookvale, Australia). Morphometry was determined using digital analysis of images (Olympus cellSens Entry 2.1 software and Olympus Digital Colour camera DP73, Olympus Corporation, Shinjuku, Tokyo, Japan). Photomicrographs of *C. ornithophilus* n. sp. oocysts observed by DIC, ACMV, ZN, AP and IFA were stored at the Institute of Parasitology, Biology Centre of the Czech Academy of Sciences, Czech Republic.

Molecular analyses

Total genomic DNA was extracted from 20,000 purified oocysts, 200 mg of faeces, or 200 mg of tissue by bead disruption for 60 s at 5.5 m/s using 0.5 mm glass beads in a FastPrep®24 Instrument (MP Biomedicals, CA, USA) followed by isolation/purification using Exgene™ Stool DNA mini (GeneAll Biotechnology Co. Ltd, Seoul, Korea) or DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions. Purified DNA was stored at −20 °C. A nested PCR approach was used to amplify a partial region of the small subunit (*SSU*) rRNA [42, 43], *actin* [44], 70 kilodalton heat-shock protein (*HSP70*) [45] and *gp60* [46–48] genes. The PCR conditions were slightly modified, for more details see [6]. Molecular grade water and DNA of *C. parvum* were used as negative and positive controls, respectively. Secondary PCR products were detected in 1.5% agarose gel stained with ethidium bromide. PCR products were cut out from gel, purified using Gen Elute Gel Extraction Kit (Sigma, St. Louis, MO, USA) and sequenced in both directions with an ABI 3130 genetic analyser (Applied Biosystems, Foster City, CA) using the secondary PCR primers in commercial laboratory (SEQme, Dobříš, Czech Republic).

Phylogenetic analyses

The nucleotide sequences obtained in this study were edited using the ChromasPro 2.4.1 software (Technelysium, Pty, Ltd., South Brisbane, Australia) and aligned with reference sequences downloaded from GenBank using MAFFT version 7 online server (http://mafft.cbrc.jp/alignment/software/). The most appropriate evolutionary models for phylogeny analyses and values of all parameters for each model were selected using the MEGAX software [49, 50]. The evolutionary history was inferred by using the Maximum Likelihood (ML) method based on the Tamura 3-parameter model [51] selected for *SSU* and *HSP70* alignments and the general time reversible model [52] was selected for actin alignment. The trees with the highest log likelihood were shown. Bootstrap support for branching was based on 1000 replications. Phylogenetic trees obtained from the MEGAX (https://www.megasoftware.net/) were edited in CorelDrawX7 (https://www.coreldraw.com). Sequences of *SSU* (MN969954-MN969968), *actin* (MN973944-MN973958), *HSP70* (MN973934-MN973943) and *gp60* (MN973959-MN973963) generated in this study were deposited in the GenBank database.

Animals for transmission studies

Five adult cockatiels (*Nymphicus hollandicus* (Kerr)), five one-day-old chickens, five one-day-old geese (*Anser anser* f. *domestica* L.), five seven-day- and eight-week-old SCID mice (*Mus musculus*; strain C.B-17) and five seven-day and eight-week-old BALB/c mice were used for transmission studies. Three adult cockatiels, chickens, geese and seven-day and eight-week-old SCID and BALB/c mice used as a negative control. As a control, the infectivity of *C. parvum* from a naturally infected Holstein calf for three adult cockatiels, chickens, geese and seven-day and eight-week-old SCID and BALB/c mice was verified. All animals, except chickens, geese and seven-day-old mice, which were hatched under laboratory conditions, were screened every other day for the presence of oocysts of *Cryptosporidium* spp. and specific DNA two weeks prior to transmission studies. Cockatiels originated from breeders located in the Czech Republic and laboratory mice were obtained from Charles River (Germany).

Animal care

Rodents were individually housed in ventilated cages (Tecniplast, Buguggiate, Italy). Chickens and geese were housed in boxes and cockatiels were kept in separate aviaries. The size of boxes and aviaries were according to regulated by Czech legislation (Act No 246/1992 Coll., on protection of animals against cruelty). An external source of heat was used in the first five days for chickens and geese. Sterilized diet and water were available for all animals *ad libitum*. Animal caretakers wore sterile shoe covers and disposable coveralls and disposable gloves always they entered the experimental room. Wood-chip bedding and disposable protective clothing were removed from the experimental room and incinerated.

Experimental design

A total 20,000 purified oocysts of *C. ornithophilus* n. sp., suspended in 10 µl of distilled water, were dropped into the mouth/beak of each animal. Animals serving as negative controls were inoculated orally with 10 µl of distilled water. Faecal samples from all animals were screened daily for the presence of *Cryptosporidium* oocysts using ACMV staining and the presence of *Cryptosporidium*-specific DNA was confirmed using nested PCR/sequencing targeting the *SSU* gene. All experiments were terminated 30 days post-infection (dpi). Infection intensity was reported as the number of oocysts per gram (opg) of faeces, as previously described by Kváč et al. [53]. In addition, faecal consistency and colour and general health status were examined daily. The sequence identity of the *Cryptosporidium* DNA recovered from infected hosts to inoculum and original isolate at *SSU*, *actin* and *HSP70* was verified in each experimentally infected animal.

Histopathological and scanning electron microscopy (SEM) examinations

Two animals from each group (at 10 and 20 dpi) were examined at necropsy. Tissue samples from oesophagus; stomach in rodents and proventriculus and ventriculus in birds; duodenum; jejunum (proximal, central and distal); ileum; caecum and colon were collected for histology and SEM followed by processing according [6]. Slides for histology were examined at 100–400× magnification and documented using Olympus cell Sens Entry 2.1 (Olympus Corporation, Shinjuku, Tokyo, Japan) equipped with a digital camera (Olympus DP73). Samples for SEM were examined using a JEOL JSM-7401F-FE SEM and documented using ETD Detector A PRED (Thermo Fisher Scientific, Waltham, MA, USA). Additionally, DNA from tissue samples was isolated and the sequence identity to inoculum and original isolate at *SSU*, *actin* and *HSP70* was verified.

Staining of mucosal smears

Wright staining procedures were used to visualize *Cryptosporidium* spp. developmental stages in the gastrointestinal tract of chickens [54]. Tissue samples of the large intestine (selected on the basis of histological examination) were washed with cold PBS with subsequent exposure to serum from *Cryptosporidium*-negative

chickens for five min. The mucous membrane was gently scrapped with a scalpel and smeared on a glass slide. Wet mucosal smears were fixed with osmium vapour for 15 min followed by Wright staining for 6 min. Slides were viewed at 1000× magnification and documented using Olympus cell Sens Entry 2.1 (Olympus Corporation, Shinjuku, Tokyo, Japan) equipped with a digital camera (Olympus DP73).

Statistical analysis

Differences in *Cryptosporidium* spp. oocysts size were tested using Hotelling's multivariate version of the 2 sample t-test, *package ICSNP: Tools for Multivariate Nonparametrics* in R 4.0.0. [55]. The hypothesis tested was that two-dimensional mean vectors of measurement are the same in the two populations being compared.

Results

A total of 164 juvenile and 40 adult ostriches were screened for the presence of *Cryptosporidium* infection. *Cryptosporidium* spp. was detected on three out of four ostrich farms. Out of 204 faecal samples, five (2.5%) were microscopically positive for the presence of *Cryptosporidium* oocysts and 12 (5.9%) contained specific DNA of *Cryptosporidium* spp. (Table 2). All microscopically positive samples were also positive for *Cryptosporidium* DNA. Only juvenile ostriches ($n=12$) were infected with *Cryptosporidium* spp. Screened animals had good health and faecal consistency appropriate to the age of birds and feeding.

All birds positive for *Cryptosporidium*-specific DNA were successfully genotyped by sequence analysis of *SSU* and *actin* genes (Table 2). ML trees constructed from *SSU* and *actin* sequences in this study showed the presence of *C. ubiquitum* Fayer, Santín & Macarisin, 2010 ($n=5$) and *C. ornithophilus* n. sp. ($n=7$; Table 2, Figs. 1, 2). *HSP70* gene sequences were successfully amplified only from samples positive for *C. ornithophilus* n. sp. (Fig. 3). The *C. ubiquitum gp60* gene was amplified and sequenced from five positive DNA samples from farm no. 1 (Table 2, Fig. 4). Sequences were identical to each other and clustered with subtype family XIIa (Fig. 4). Out of seven ostriches positive for *C. ornithophilus* n. sp., five shed microscopically detectable oocysts (6000–18,000 opg, Table 2). Birds positive for *C. ubiquitum* DNA did not shed oocysts detectable by microscopy.

Cryptosporidium ornithophilus n. sp. oocysts did not infect 7-day-old and 8-week-old BALB/c or SCID mice, whereas 7-day-old BALB/c and both age categories of SCID mice were infected with *C. parvum* (control group, data not shown). All chickens, geese and cockatiels inoculated with oocysts of *C. ornithophilus* n. sp. developed infections. Oocysts or specific DNA were first detected at 4 dpi, 7 dpi and 8 dpi in geese, chickens and cockatiels, respectively (Fig. 5). The infection intensity ranged from 2000 to 16,000 opg in chickens and cockatiels and from 1000 to 8000 opg in geese (Fig. 5).

Molecular, histological and SEM analyses and examination of stained mucosal smears of gastrointestinal tract tissue in birds with *C. ornithophilus* n. sp. showed

Table 2 *Cryptosporidium* species and genotypes from this study, detected by amplification of small subunit ribosomal rRNA (*SSU*), *actin*, 70 kDa heat-shock protein (*HSP70*) and 60 kDa glycoprotein (*gp60*) gene fragments in juvenile common ostriches (*Struthio camelus*) on commercial farms in the Czech Republic

Farm No.	No. of positive/no. of screened	ID of positive animal	Microscopical positivity (opg)	Genotyping at the gene loci			
				SSU	*Actin*	*HSP70*	*gp60*
1	5/40	43201	No	*C. ubiquitum*	*C. ubiquitum*	–	XIIa
		43205	No	*C. ubiquitum*	*C. ubiquitum*	–	XIIa
		43210	No	*C. ubiquitum*	*C. ubiquitum*	–	XIIa
		43223	No	*C. ubiquitum*	*C. ubiquitum*	–	XIIa
		43228	No	*C. ubiquitum*	*C. ubiquitum*	–	XIIa
2	0/64	–	–	–	–	–	–
3	3/50	44782	Yes (8000)	*C. ornitophilus* n. sp.	*C. ornitophilus* n. sp.	*C. ornitophilus* n. sp.	–
		44790	No	*C. ornitophilus* n. sp.	*C. ornitophilus* n. sp.	*C. ornitophilus* n. sp.	–
		44796	Yes (12,000)	*C. ornitophilus* n. sp.	*C. ornitophilus* n. sp.	*C. ornitophilus* n. sp.	–
4	4/50	43545	Yes (12,000)	*C. ornitophilus* n. sp.	*C. ornitophilus* n. sp.	*C. ornitophilus* n. sp.	–
		43551	No	*C. ornitophilus* n. sp.	*C. ornitophilus* n. sp.	*C. ornitophilus* n. sp.	–
		43587	Yes (6000)	*C. ornitophilus* n. sp.	*C. ornitophilus* n. sp.	*C. ornitophilus* n. sp.	–
		43588	Yes (18,000)[a]	*C. ornitophilus* n. sp.	*C. ornitophilus* n. sp.	*C. ornitophilus* n. sp.	–

[a] Animal serving as a source of oocysts for transmission studies

Note: Infection intensity of *Cryptosporidium* spp. is expressed as the number of oocysts per gram of faeces (opg)

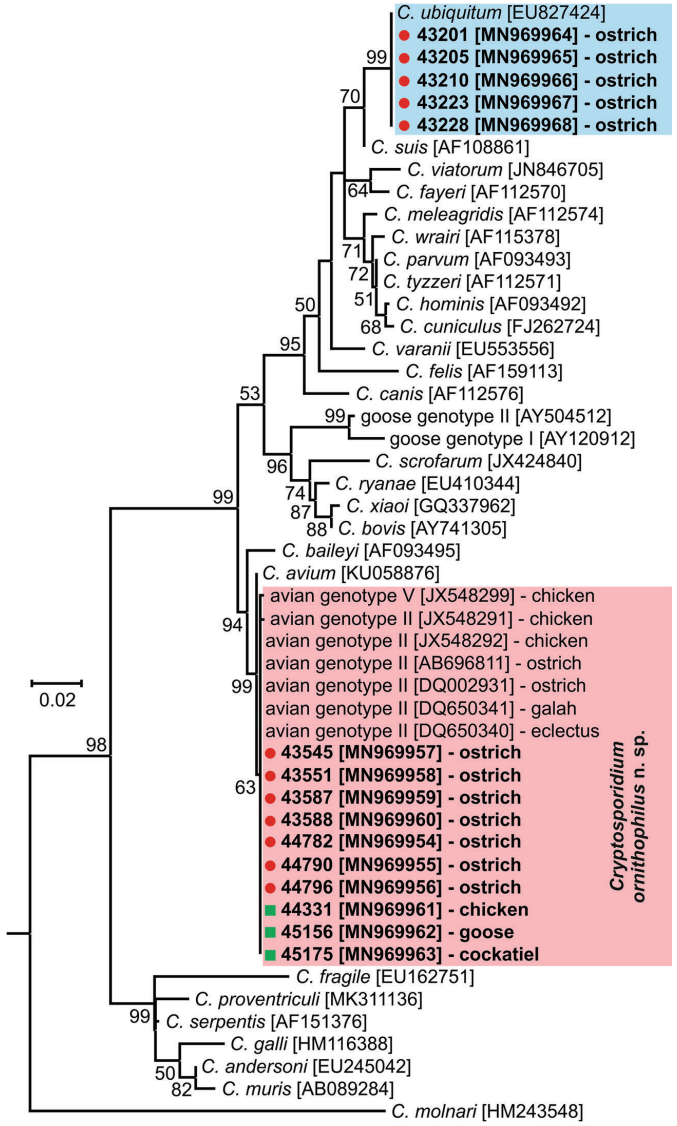

Fig. 1 Maximum likelihood tree (— ln = 3130.05) based on partial sequences of the gene encoding the small subunit rRNA (*SSU*), including sequences obtained in this study from naturally (red circle and bolded) and experimentally (green square and bolded) infected hosts. Tamura's 3-parameter model was applied, using a discrete Gamma distribution and invariant sites. The robustness of the phylogeny was tested with 1000 bootstrap pseudoreplicates and numbers at the nodes represent the bootstrap values > 50%. The scale-bar indicates the number of substitutions per site. Sequences obtained in this study are identified by isolate number (e.g. 43201)

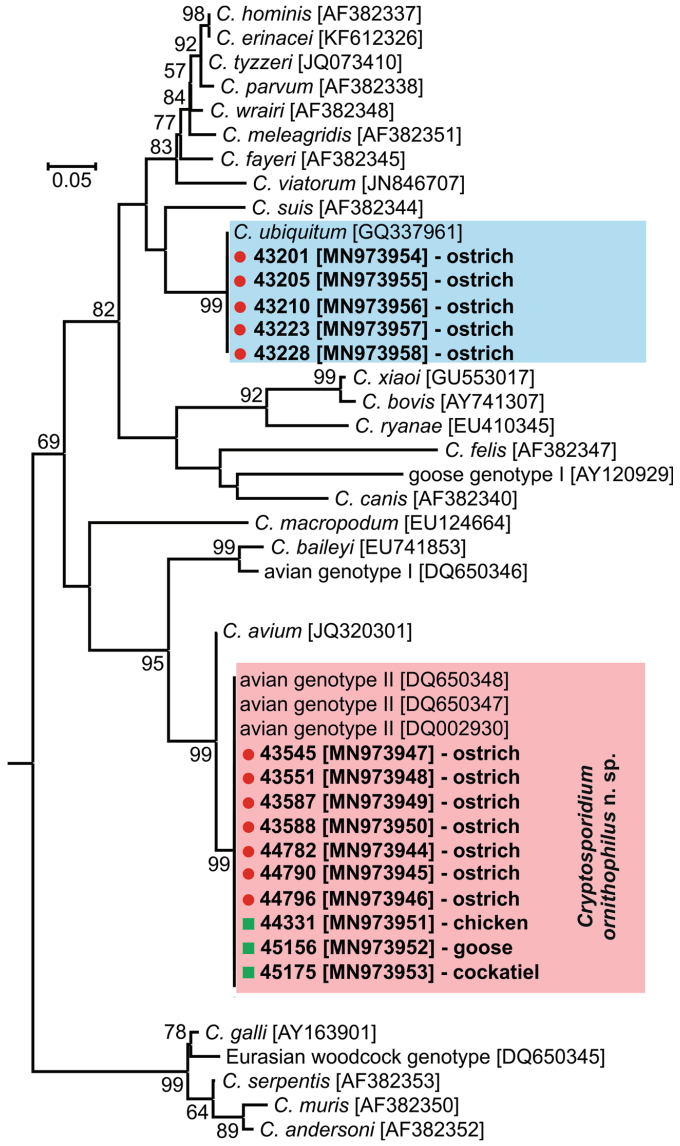

Fig. 2 Maximum likelihood tree (− ln = 3641.49) based on partial sequences of the actin gene, including sequences obtained in this study from naturally (red circle and bolded) and experimentally (green square and bolded) infected hosts. The General Time Reversible model was applied, using a discrete Gamma distribution and invariant sites. The robustness of the phylogeny was tested with 1000 bootstrap pseudoreplicates and numbers at the nodes represent the bootstrap values > 50%. The scale-bar indicates the number of substitutions per site. Sequences obtained in this study are identified by isolate number (e.g. 43201)

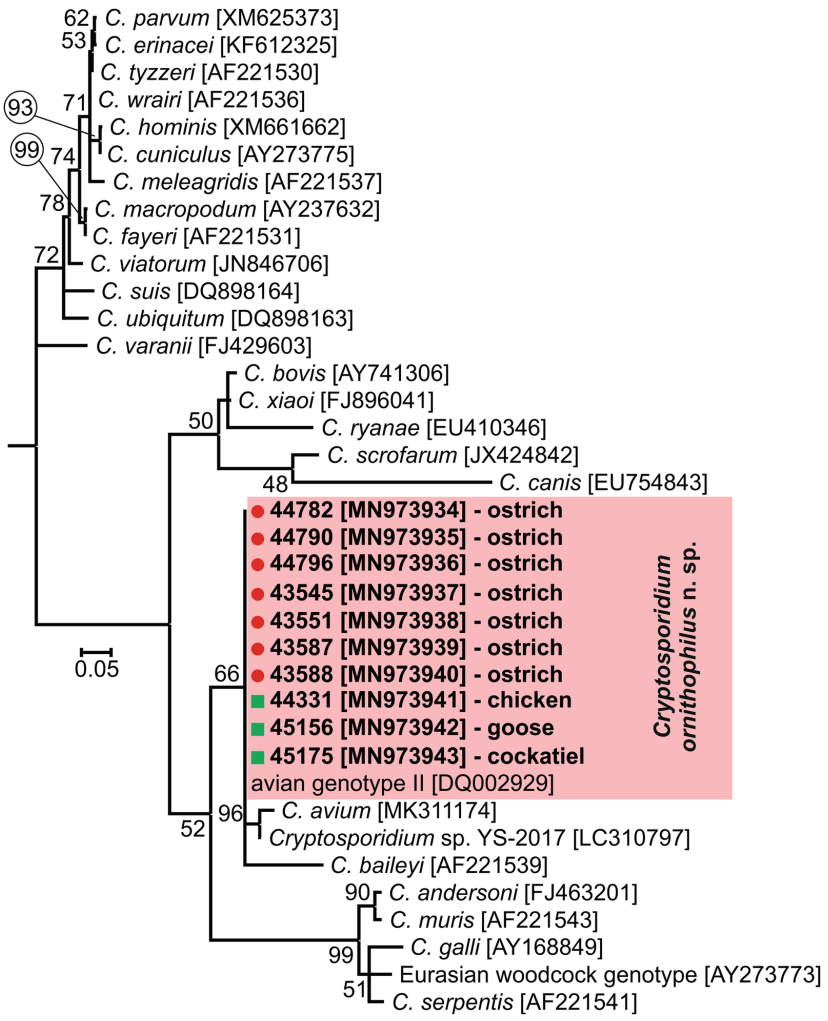

Fig. 3 Maximum likelihood tree (— ln = 2009.56) based on partial sequences of the 70 kDa heat-shock protein gene, including sequences obtained in this study from naturally (red circle and bolded) and experimentally (green square and bolded) infected hosts. Tamura's 3-parameter model was applied, using a discrete Gamma distribution and invariant sites. The robustness of the phylogeny was tested with 1000 bootstrap pseudoreplicates and numbers at the nodes represent the bootstrap values > 50%. The scale-bar indicates the number of substitutions per site. Sequences obtained in this study are identified by isolate number (e.g. 43201)

the presence of developmental stages only in the caecum and colon of chickens and geese sacrificed 10 and 20 dpi (Figs. 6, 7). Few developmental stages were detected on each villus (Figs. 6, 7). Developmental stages were not detected in cockatiels, but specific DNA was detected exclusively in the caudal part of the ileum.

The morphometry of the developmental stages of *C. ornithophilus* n. sp. was examined in preparations with Wright's stain (Table 3). Most of the detected developmental stages were enveloped by a parasitophorous sac, which appeared as an unstained halo (Fig. 8). A large number of oocysts was detected, and most were

unstained with sporozoites not visible (Fig. 8). We were not able to differentiate between thin- and thick-walled oocysts. Free sporozoites were not detected, but a photomicrograph of sporozoites following oocyst excystation is included in Fig. 8. Mononuclear trophozoites were the most frequently observed developmental stage which also showed a high variability in size (Fig. 8; Table 3). Type I meronts, containing 8 merozoites, were observed frequently (Fig. 8), while Type II meronts, with 4 merozoites, were found rarely (Fig. 8). Free merozoites were found rarely (Fig. 8). Microgamonts were found rarely (Fig. 8), but macrogamonts, typified by a number of amylopectin granules in their cytoplasm and a foam-like appearance, were frequently observed (Fig. 8). Zygotes were lightly stained compared to the unstained oocysts (Fig. 8).

SSUI, *actin* and *HSP70* sequences obtained from the original isolate of *C. ornithophilus* n. sp. (ostrich) were identical to isolates recovered from faeces of chicken 0 and all other birds infected during the whole experiment. Additionally, sequences obtained from the tissue specimens of caecum and colon of chickens and geese and in the ileum of cockatiels were also identical to the inoculum. The gene encoding *gp60* was not successfully amplified in any animal experimentally infected with *C. ornithophilus* n. sp., indicating the absence of *C. ubiquitum* or other species and genotypes of *Cryptosporidium* spp. (e.g. *C. parvum*) that could be part of the inoculum.

The above data tend to justify the distinct status of *Cryptosporidium ornitophilus* n. sp., which is described below.

Family Cryptosporidiidae Léger, 1911
Genus *Cryptosporidium* Tyzzer, 1907

***Cryptosporidium ornitophilus* n. sp.**

Syn. *Cryptosporidium* sp. ex *Struthio camelus* 2005 of Meireles et al. [59]; *Cryptosporidium* avian genotype II of Ng et al. [7], Nguyen et al. [36] and Sevá et al. [56]

Type-host: *Struthio camelus* Linnaeus (Struthioniformes: Struthionidae), common ostrich.

Other natural hosts: Alexandrine (*Psittacula eupatria* (L.)) (as *Cryptosporidium* avian genotype II [7]), chicken (*Gallus gallus* f. *domestica*) (as *Cryptosporidium* avian genotype II [57]), cockatiel (*Nymphicus hollandicus*) (as *Cryptosporidium* avian genotype II [7]), eclectus (*Eclectus roratus* (Müller)) (as *Cryptosporidium* avian genotype II [7]), galah (*Eolophus roseicapilla* (Vieillot)) (as *Cryptosporidium* avian genotype II [7]), Major Mitchell cockatoo (*Cacatua leadbeateri* (Vigors)) (as *Cryptosporidium* avian genotype II [7]), princess parrots (*Polytelis alexandrae* (Gould)) (as *Cryptosporidium* avian genotype II [7]), sun conure (*Aratinga solstitialis* (L.)) (as *Cryptosporidium* avian genotype II [7]), white-eyed parakeet (*Aratinga leucophthalma* (Statius Müller)) (as *Cryptosporidium* avian genotype II [56]).

Experimentally susceptible host: *Gallus gallus* f. *domestica* L. (Galliformes: Phasianidae), chicken; *Anser anser* f. *domestica* L. (Anseriformes: Anatidae), goose; *Nymphicus hollandicus* (Kerr) (Psittaciformes: Cacatuidae), cockatiel.

Type-locality: Ostrich farm at Židovice (50.4451578N, 14.2297606E), Czech Republic.

Other locality: Ostrich farm at Fulnek (49.7123761N, 17.9031931E) Czech Republic.

Type-material: Tissue samples in 10% formaldehyde and histological sections of infected cecum (no. 2/2019) and colon (no. 3/2019); genomic DNA isolated from faecal samples of naturally (isolation no. 43545) and experimentally (isolation no. 44331) infected chicken; genomic DNA isolated from cecum and colon of experimentally infected chicken (isolation no. 44331); hapantotypes: digital photomicrographs nos. DIC 1-13/43545, ACMV 1-11/43545, IF 1-9/43545, AP 1-12/43545, ZN IF 1-8/43545, PAS 2-3/2019 and SEM 744.75-744.79 and 745.68-745.74) and faecal smear slides with oocysts stained by ACMV staining from experimentally infected chicken (nos. 10/44331, 11/44331 and 12/44331). Specimens deposited at the Institute of Parasitology, Biology Centre of the Czech Academy of Sciences, Czech Republic.

Site of infection: Caecum, colon and bursa Farbricii (present study and [31]).

Distribution: As *Cryptosporidium* sp. ex *Struthio camelus* 2005: Brazil [36] and as *Cryptosporidium* avian genotype II: Australia [7], Brazil [56], China [57] and Vietnam [36].

Prepatent period: *Gallus gallus* f. *domestica*: 7 dpi; *Nymphicus hollandicus*: 8 dpi; *Anser anser* f. *domestica*: 4 dpi.

Patent period: At least 30 dpi in all experimentally infected birds (*Gallus gallus* f. *domestica*, *Nymphicus hollandicus* and *Anser anser* f. *domestica*)

Representative DNA sequences: Representative nucleotide sequences of the *SSUI* (MN969957), *HSP70* (MN973934) and *actin* (MN973947) genes were submitted to the GenBank database.

ZooBank registration: To comply with the regulations set out in Article 8.5 of the amended 2012 version of the *International Code of Zoological Nomenclature* (ICZN) [58], details of the new species have been submitted to ZooBank. The Life Science Identifier (LSID) of the article is urn:lsid:zoobank.org:pub:593209C2-7F5B-47F9-93F3-02C81E8A747C. The LSID for the new name *Cryptosporidium ornitophilus* is urn:lsid:zoobank.org:act:FE74CF3C-6734-424B-889E-C47108DEBA60.

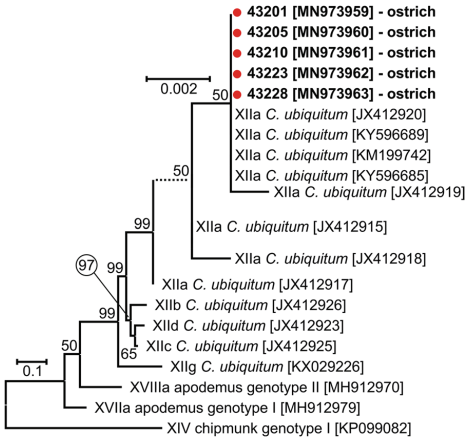

Fig. 4 Maximum likelihood tree (− ln = 4017.25) based on partial sequences of the 60 kDa glycoprotein gene of *Cryptosporidium ubiquitum*, *Cryptosporidium* apodemus genotype I and II and *Cryptosporidium* chipmunk genotype I, including sequences obtained in this study from naturally infected hosts (red circles and bolded). Tamura's 3-parameter model was applied, using invariant sites. The robustness of the phylogeny was tested with 1000 bootstrap pseudoreplicates and numbers at the nodes represent the bootstrap values > 50%. The scale-bar indicates the number of substitutions per site. Sequences obtained in this study are identified by isolate number (e.g. 43201)

Etymology: The species name is derived from the lack of host specificity among birds and its non-infectiousness to other vertebrates.

Description

Oocysts obtained from fresh feces specimens ex *Struthio camelus* ovoidal (Fig. 9), measuring 5.2–6.8 × 4.7–5.5 µm (6.1 ± 0.4 × 5.2 ± 0.2 µm) with a length/width ratio of 1.1–1.4 (1.19 ± 0.08). Oocyst wall single-layered, smooth, colorless. Micropyle and polar granule absent. Oocyst residuum present, composed of numerous small granules and one spherical globule. Four sporozoites measuring 5.5–6.6 × 0.5–0.6 µm (6.1 ± 0.3 × 0.6 ± 0.1 µm) present within each oocyst. For the measurements of other developmental stages see Table 3.

Remarks

Oocysts in faecal smears showed typical *Cryptosporidium* ACMV, Ziehl-Neelsen, AP staining characteristics (Fig. 9). Fixed *C. ornithophilus* n. sp. oocysts were detectable with a FITC conjugated anti-*Cryptosporidium* oocyst wall antibody developed primarily for *C. parvum* (Fig. 9). There were no statistically significant size differences between oocysts from naturally infected ostriches and oocysts obtained from experimentally infected chickens ($T^2 = 2.249703$, $df_1 = 2$, $df_2 = 97$, $P = 0.1109$), geese ($T^2 = 0.96185$, $df_1 = 2$, $df_2 = 97$, $P = 0.3858$) and cockatiels ($T^2 = 2.221246$, $df_1 = 2$, $df_2 = 97$, $P = 0.1139$; Table 4). Oocysts of *C. ornithophilus* n. sp. are larger than those of *C. avium* ($T^2 = 32.522$, $df_1 = 2$, $df_2 = 140$, $P < 0.0001$) and *C. parvum* ($T^2 = 147.32$, $df_1 = 2$, $df_2 = 78$, $P < 0.0001$) and smaller than *C. proventriculi* Holubová, Zikmundová, Limpouchová, Sak, Konečný, Hlásková, Rajský, Kopacz, McEvoy & Kváč, 2019 ($T^2 = 161{,}04$ $df_1 = 2$, $df_2 = 90$, $P < 0.0001$) and *C. galli* ($T^2 = 35{,}522$, $df_1 = 2$, $df_2 = 78$, $P < 0.0001$). *Cryptosporidium ornithophilus* n. sp. can be differentiated genetically from other *Cryptosporidium* spp. based on sequences of *SSU*, *actin* and *HSP70* genes and on the basis of localization of life-cycle developmental stages in the host. While other bird-specific *Cryptosporidium* spp. primarily infect the proventriculus/ventriculus (*C. proventiculi* and *C. galli*) or small intestine (*C. avium*, *C. meleagridis* and *C. baileyi*) within gastrointestinal tract, *C. ornithophilus* n. sp. infects the caecum and colon.

Table 3 Size of developmental stages of *Cryptosporidium ornitophilus* n. sp. obtained from the colon of an experimentally infected chicken (*Gallus gallus* f. *domestica*) with 200,000 oocysts and sacrificed 20 days post-infection

Developmental stage	Length (µm) Range (Mean ± SD)	Width (µm) Range (Mean ± SD)
Oocyst	5.24–6.74 (6.13 ± 0.34)	4.71–5.48 (5.21 ± 0.23)
Sporozoite	5.47–6.57 (6.07 ± 0.32)	0.54–0.63 (0.59 ± 0.02)
Trophozoite	2.56–6.40 (4.36 ± 1.16)	2.16–5.50 (3.90 ± 1.05)
Early Type I meront	4.95–6.54 (5.96 ± 0.57)	4.00–5.79 (5.12 ± 0.64)
Late Type I meront	6.66–8.94 (7.50 ± 0.84)	5.72–7.11 (6.40 ± 0.44)
Type II meront	6.60–6.78 (6.67 ± 0.10)	6.37–6.54 (6.48 ± 0.10)
Merozoite	4.61–5.41 (5.05 ± 0.11)	0.62–0.95 (0.77 ± 0.33)
Macrogamont	5.29–8.87 (6.70 ± 0.97)	4.32–8.13 (6.10 ± 1.09)
Microgamont	6.14–6.92 (6.54 ± 0.23)	6.02–6.73 (6.39 ± 0.23)
Zygote	5.56–6.83 (6.14 ± 0.45)	4.19–5.79 (5.20 ± 0.62)

Discussion

Birds are naturally parasitized with several *Cryptosporidium* species and genotypes [16, 18]. Here, we reported the occurrence of *Cryptosporidium* spp. in ostriches farmed commercially and described *Cryptosporidium* avian genotype II as a new species. Previous studies have shown that ostriches are frequently infected with *C. baileyi* [32–34] and *C. ornithophilus* n. sp. [19, 36, 59]; however, we detected *C. ornithophilus* n. sp. and *C. ubiquitum*. The absence of *C. baileyi* could be explained by the age of the birds screened in the present study. Previous studies

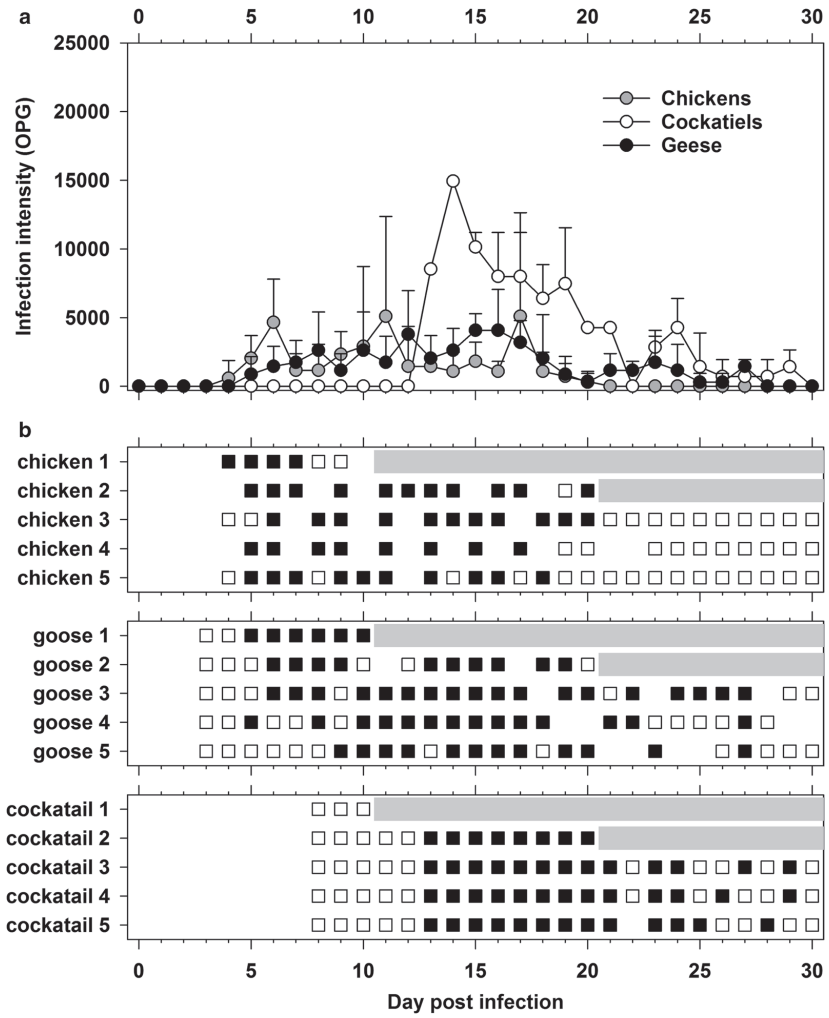

Fig. 5 Course of infection of *Cryptosporidium ornitophilus* n. sp. in experimentally infected chickens (*Gallus gallus* f. *domestica*), geese (*Anas platyrhynchos* f. *domestica*) and cockatiels (*Nymphicus hollandicus*). **a** Infection intensity as number of oocysts per gram of faeces (opg). **b** Daily shedding of *C. ornitophilus* n. sp. based on coprological and molecular examination of faeces. Open squares indicate detection of specific DNA; filled squares indicate detection of oocysts by microscopy; grey rectangles indicate sacrifice and dissection of animal

reported *C. baileyi* in ostriches younger than 3 months with older birds being infected rarely or not at all [32, 34]. In this study, the occurrence of *C. ornithophilus* n. sp. in birds aged 9–14 months was 4.3% (7/164), which is similar to that reported in Vietnamese ostriches older than 12 months (5.8%; [36]). The absence of *C. ornithophilus* n. sp. in birds older than three years in this study could be due to age-related resistance or immunity, as described for *C. baileyi*, *C. avium*, *C. parvum*, *C. muris* and *C. andersoni* Lindsay, Upton, Owens, Morgan, Mead & Blagburn, 2000 in various hosts [60–62], but this needs to be examined experimentally.

Cryptosporidium ubiquitum is not typically found in birds so our finding of five ostriches on a single farm

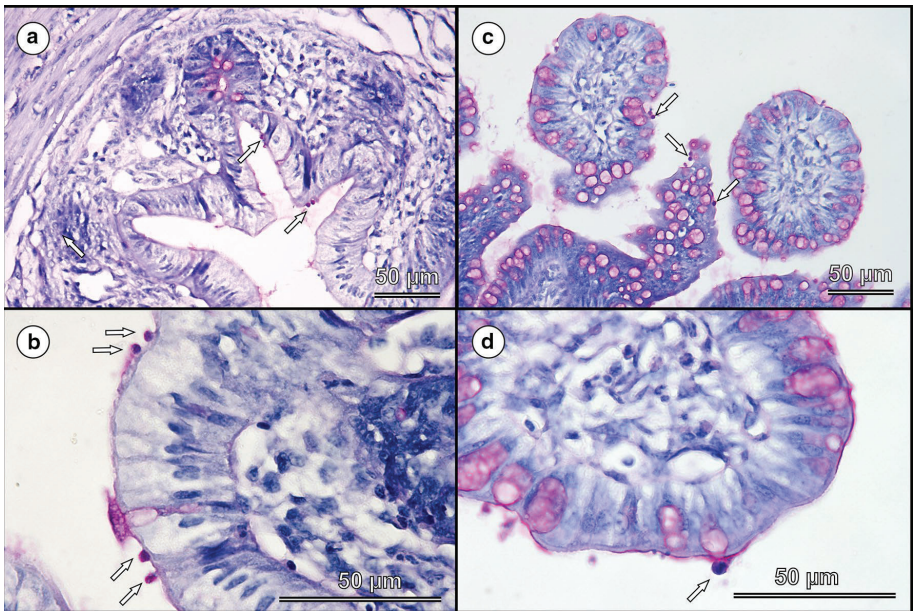

Fig. 6 Histology sections of the caecum (**a** and **b**) and colon (**c** and **d**) of a chicken (*Gallus gallus* f. *domestica*) experimentally infected with 20,000 oocysts of *Cryptosporidium ornitophilus* n. sp., sacrificed 10 days post-infection. Attached developmental stages of *C. ornitophilus* n. sp. are indicated by arrows. Periodic Acid-Schiff (PAS) staining. *Scale-bars*: 50 µm

positive for this species was unexpected. Li et al. [63] also detected *C. ubiquitum* in birds (common hill mynas, *Gracula religiosa* L.) at commercial markets in China. It is possible that the detected DNA was due to mechanical passage, not an active infection. The cohabitation of livestock, companion and wild animals can result in *Cryptosporidium* oocyst passage through non-susceptible animals without establishing infection [64–66]. We cannot exclude that some wild animals may be the source of *C. ubiquitum*. Our failure to detect oocysts also suggests that any infection was likely to be of low intensity.

Five avian *Cryptosporidium* spp. (*C. avium*, *C. baileyi*, *C. galli*, *C. meleagridis* and *C. proventriculi*) have been recognized to date, and these differ in host range, oocyst morphometry, predilection sites and course of infection. The mean size of *C. ornithophilus* n. sp. oocysts from this study (6.1 × 5.1 µm) was similar to those reported as *Cryptosporidium* avian genotype II (6.0 × 4.8 µm) by Santos et al. [31] and Meireles et al. [59], and the oocysts are morphometrically indistinguishable from those of *C. baileyi* (6.3 × 4.6 µm) [2] and *C. avium* (6.3 × 4.9 µm) [5]. Oocysts of *C. ornithophilus* n. sp. are smaller than those of *C. proventriculi* (8.4 × 6.7 µm) [6] and *C. galli* (8.3 × 6.3 µm) [4] and larger than those of *C. meleagridis* (5.0 × 4.3 µm) [3]. *Cryptosporidium ornithophilus* n. sp. infects the caecum, colon and bursa Fabricii. *Cryptosporidium baileyi* also infects the caecum, colon and bursa Fabricii (in addition to other sites in the intestine and lungs) and *C. avium* also infects the caecum (in addition to the ileum) and their oocysts are similar in size to *C. ornithophilus* n. sp. [2, 5, 31], which would make it difficult to distinguish infections without the use of molecular tools. In addition to *C. ornithophilus* n. sp., *C. baileyi* and *C. avium*, *C. meleagridis* may also develop in the colon [67, 68], but these species could be distinguished based on oocyst size. In contrast to *C. baileyi* and *C. avium*, *C. ornithophilus* n. sp. did not develop at extraintestinal sites [5, 61, 69, 70].

Similar to Ng et al. [7] and Meireles et al. [59], we found no obvious clinical symptoms or mortality in birds naturally or experimentally infected with *C. ornithophilus* n. sp. There have been reports of clinical cryptosporidiosis, including prolapse of the phallus and cloaca, enteritis and pancreatitis, in ostrich chickens, but the isolates were not genotyped [21–23, 29–31] and other species, such as *C. baileyi*, may have been the cause of disease.

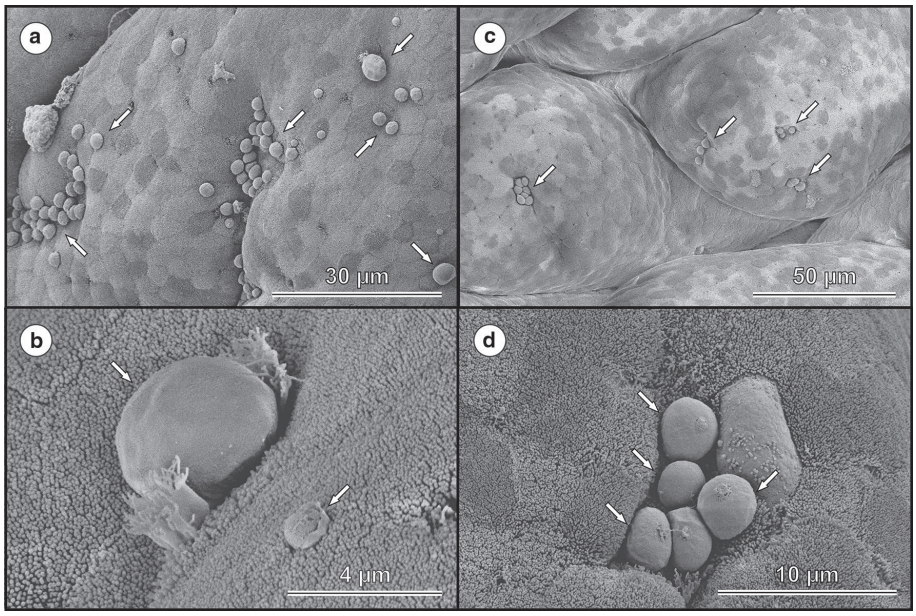

Fig. 7 Scanning electron micrographs of developmental stages of *Cryptosporidium ornitophilus* n. sp. (arrows) on the epithelia surface of the caecum (**a** and **b**) and colon (**c** and **d**) of a chicken (*Gallus gallus* f. *domestica*) experimentally infected with 20,000 oocysts and sacrificed 10 days post-infection

Although *C. ornithophilus* n. sp. has been reported most frequently in ostriches, reports of natural and experimental infections in alexandrine, chickens, cockatiels, eclectus, galah, geese, Major Mitchell cockatoo, princess parrots, sun conure and white-eyed parakeet suggests a broad host range [7, 19, 56, 71]. The prepatent period of *C. ornithophilus* n. sp. (4–8 dpi) is similar to *C. meleagridis*, *C. baileyi* and *C. proventriculi* [6, 72–75].

Phylogenetic analyses based on *SSU*, *actin* and *HSP70* gene sequences showed that *C. ornithophilus* n. sp. is genetically distinct from known species and is most closely related to *C. baileyi* and *C. avium*. At the *SSU* locus, *C. ornithophilus* n. sp. shares 92.8% and 93.5% similarity with *C. baileyi* and *C. avium*, respectively. This is comparable to the similarity between *C. andersoni* and *C. ryanae* (91.1%) or *C. muris* and *C. suis* (93.3%). At the *actin* locus, similarities with *C. baileyi* and *C. avium* are 88.7% and 98.1%, respectively. In comparison, *C. bovis* and *C. ryanae* share 88.1% similarity and *C. parvum* and *C. erinacei* share 98.3% similarity at the *actin* locus. At the *HSP70* locus, *C. ornithophilus* n. sp. shares 91.3% and 95.6% similarity with *C. baileyi* and *C. avium*, respectively. In comparison, *C. parvum* and *C. erinacei* share 99.2% similarity at the *HSP70* locus.

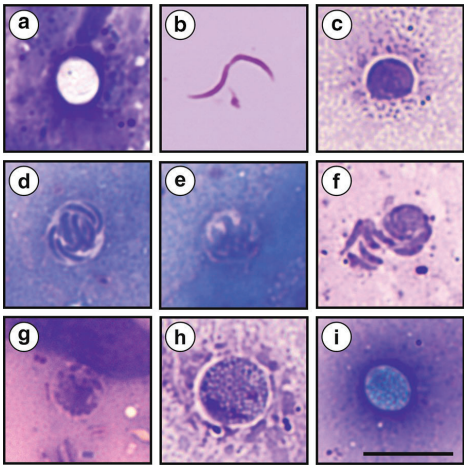

Fig. 8 Developmental stages of *Cryptosporidium ornitophilus* n. sp. obtained from the colon of chickens (*Gallus gallus* f. *domestica*) experimentally infected with 20,000 oocysts and sacrificed 10 days post-infection. **a** Oocyst. **b** Sporozoite. **c** Mononuclear trophozoite. **d** Type I meront. **e** Type II meront. **f** Merozoites. **g** Microgamont. **h** Macrogamont. **i** Zygote. *Scale-bar*: 10 µm

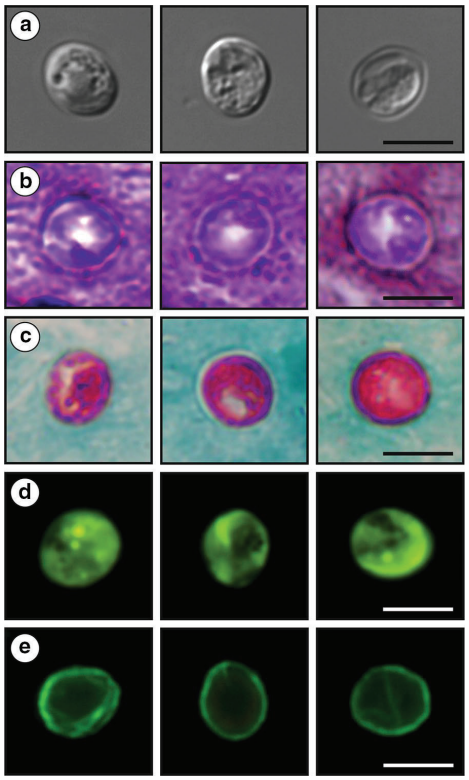

Fig. 9 Oocysts of *Cryptosporidium ornitophilus* n. sp. visualized in various preparations. **a** Differential interference contrast microscopy. **b** Aniline-carbol-methyl violet staining. **c** Ziehl-Nielsen staining. **d** Auramine-phenol staining. **e** Labelled with anti-*Cryptosporidium* FITC-conjugated antibody. *Scale-bars*: 5 µm

Cryptosporidium ornithophilus n. sp. represents the 44th valid species within the genus *Cryptosporidium* (*C. alticolis* Horčičková, Čondlová, Holubová, Sak, Květoňová, Hlásková, Konečný, Sedláček, Clark, Giddings, McEvoy & Kváč, 2019, *C. andersoni*, *C. apodemi* Čondlová, Horčičková, Sak, Květoňová, Hlásková, Konečný, Stanko, McEvoy & Kváč, 2018, *C. avium*, *C. bailey*, *C. bovis* Fayer, Santín & Xiao, 2005, *C. canis* Fayer, Trout, Xiao, Morgan, Lai & Dubey, 2001, *C. cichlidis* Paperna & Vilenkin, 1996, *C. cuniculus* Robinson, Wright, Elwin, Hadfield, Katzer & Bartley 2010, *C. ditrichi* Čondlová, Horčičková, Sak, Květoňová, Hlásková, Konečný, Stanko, McEvoy & Kváč, 2018, *C. ducismarci* Traversa, 2010, *C. erinacei* Kváč, Hofmannová, Hlásková, Květoňová, Vítovec, McEvoy & Sak, 2014, *C. fayeri* Ryan, Power & Xiao, 2008, *C. felis* Iseki, 1979, *C. fragile* Jirků, Valigurová, Koudela, Křížek, Modrý & Šlapeta, 2008, *C. galli*, *C. homai* Zahedi, Durmic, Gofton, Kueh, Austen, Lawson, Callahan, Jardine & Ryan, 2017, *C. hominis* Morgan-Ryan, Fall, Ward, Hijjawi, Sulaiman, Fayer, Thompson, Olson, Lal & Xiao, 2002, *C. huwi* Ryan, Paparini, Tong, Yang, Gibson-Kueh, O'Hara, Lymbery & Xiao, 2015, *C. macropodum* Power & Ryan, 2008, *C. meleagridis*, *C. microti* Horčičková, Čondlová, Holubová, Sak, Květoňová, Hlásková, Konečný, Sedláček, Clark, Giddings, McEvoy & Kváč, 2019, *C. molnari* Alvarez-Pellitero & Sitjà-Bobadilla, 2002, *C. muris* Tyzzer, 1910, *C. nasoris* Hoover, Hoerr & Carlton, 1981, *C. occultus* Kváč, Vlnatá, Ježková, Horčičková, Konečný, Hlásková, McEvoy & Sak, 2018, *C. parvum* Tyzzer, 1912, *C. proliferans* Kváč, Havrdová, Hlásková, Daňková, Kanděra, Ježková, Vítovec, Sak, Ortega, Xiao, Modrý, Chelladurai, Prantlová & McEvoy, 2016, *C. proventriculi*, *C. reichenbachklinkei* Paperna & Vilenkin, 1996, *C. rubeyi* Li, Pereira, Larsen, Xiao, Phillips, Striby, McCowan & Atwill 2015, *C. ryanae* Fayer, Santin & Trout, 2008, *C.*

Table 4 Size of *Cryptosporidium ornitophilus* n. sp. obtained from naturally infected common ostriches (*Struthio camelus*) and experimentally infected chickens (*Gallus gallus* f. *domestica*), geese (*Anas platyrhynchos* f. *domestica*) and cockatiels (*Nymphicus hollandicus*)

Host	Length (µm) Range (Mean ± SD)	Width (µm) Range (Mean ± SD)	Length/width ratio Range (Mean ± SD)
Ostrich[a]	5.24–6.77 (6.13 ± 0.35)	4.68–5.47 (5.15 ± 0.24)	1.06–1.36 (1.19 ± 0.08)
Chicken[b]	5.24–6.74 (6.13 ± 0.34)	4.71–5.48 (5.21 ± 0.23)	1.08–1.32 (1.20 ± 0.09)
Goose[b]	5.28–6.67 (6.22 ± 0.31)	4.69–5.52 (5.19 ± 0.24)	1.11–1.29 (1.18 ± 0.10)
Cockatiel[b]	5.31–6.58 (6.17 ± 0.29)	4.92–5.48 (5.19 ± 0.24)	1.09–1.28 (1.21 ± 0.12)

[a] Natural infection

[b] Experimental infection

Note: Length and width of 50 oocysts from each isolate were measured under DIC at 1000× magnification, and these were used to calculate the length-to-width ratio of each oocyst

scophthalmi Alvarez-Pellitero, Quiroga, Sitjà-Bobadilla, Redondo, Palenzuela, Pardós, Vázquez & Nieto, 2004, *C. scrofarum* Kváč, Kestřánová, Pinková, Květoňová, Kalinová, Wagnerová, Kotková, Vítovec, Ditrich, McEvoy, Stenger & Sak, 2013, *C. serpentis* Levine, 1980, *C. suis* Ryan, Monis, Enemark, Sulaiman, Samarasinghe, Read, Buddle, Robertson, Zhou, Thompson & Xiao, 2004, *C. testudinis* Ježková, Horčičková, Hlásková, Sak, Květoňová, Novák, Hofmannová, McEvoy & Kváč, 2016, *C. tyzzeri* Ren, Zhao, Zhang, Ning, Jian, Wang, Lv, Wang, Arrowood & Xiao, 2012, *C. ubiquitum*, *C. varanii* Pavlásek, Lávisková, Horák, Král & Král, 1995, *C. viatorum* Elwin, Hadfield, Robinson, Crouch & Chalmers, 2012, *C. wrairi* Vetterling, Jervis, Merrill & Sprinz, 1971 and *C. xiaoi* Fayer & Santín, 2009).

Conclusions

Morphological, genetic and biological data support the establishment of *Cryptosporidium* avian genotype II as a new species, *Cryptosporidium ornithophilus* n. sp.

Abbreviations

ACMV: aniline-carbol-methyl violet; AP: auramine phenol; BSA: bovine serum albumin; DIC: differential interference contrast; DNA: deoxyribonucleic acid; dpi: days post-infection; FITC: fluorescein isothiocyanate; gp60: 60-kDa glycoprotein gene; HSP70: 70-kDa heat-shock protein; ICZN: International Commission on Zoological Nomenclature; IFA: immunofluorescence assay; ML: maximum likelihood; opg: oocysts per gram; PAS: periodic acid-schiff; PCR: polymerase chain reaction; SD: standard deviation; SCID: Severe combined immunodeficiency; SSU: small subunit rRNA; ZN: Ziehl-Neelsen.

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Authors' contributions

NH, MK and BS conceptualised the project. NH, AH and BS collected the samples. NH, MK and AH carried out the research. MK, NH and JM performed phylogenetic analysis. LT and RK performed histology, electron and light microscopy analysis. BS, LT and NH took care of experimental animals. MK, NH and JM wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

All type material and datasets on which the conclusions of the manuscript rely, are stored in the Institute of Parasitology, Biology Centre, Czech Academy of Sciences, České Budějovice, Czech Republic. Representative nucleotide sequences generated in this study were submitted to the GenBank database under the accession numbers MN969954-MN969968 and MN973934-MN973963.

Ethics approval and consent to participate

All experimental procedures complied with the laws of the Czech Republic (Act No. 246/1992 Coll., on the protection of animals against cruelty). The study design was approved by the ethical committees at the Biology Centre of CAS, the State Veterinary Administration, and the Central Commission for Animal Welfare under protocols Nos 115/2013, 35/2018 and MZP/2019/630/1411.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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7.7 Příloha VII

A chicken embryo model for the maintenance and amplification of *Cryptosporidium parvum* and *Cryptosporidium baileyi* oocysts.

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A chicken embryo model for the maintenance and amplification of *Cryptosporidium parvum* and *Cryptosporidium baileyi* oocysts

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Abstract

Cryptosporidium is a genus of apicomplexan parasites that inhabit the respiratory and gastrointestinal tracts of vertebrates. Research of these parasites is limited by a lack of model hosts. This study aimed to determine the extent to which infection at the embryo stage can enhance the propagation of *Cryptosporidium* oocysts in chickens. Nine-day-old chicken embryos and one-day-old chickens were experimentally infected with different doses of *Cryptosporidium baileyi* and *Cryptosporidium parvum* oocysts. Post hatching, all chickens had demonstrable infections, and the infection dose had no effect on the course of infection. Chickens infected as embryos shed oocysts immediately after hatching and shed significantly more oocysts over the course of the infection than chickens infected as one-day-olds. In chickens infected as embryos, *C. baileyi* was found in all organs except the brain whereas, *C. parvum* was only found in the gastrointestinal tract and trachea. In chickens infected as one-day-olds, *C. baileyi* was only found in the gastrointestinal tract and trachea. Chickens infected as embryos with *C. baileyi* died within 16 days of hatching. All other chickens cleared the infection. Infection of chickens as embryos could be used as an effective and simple model for the propagation of *C. baileyi* and *C. parvum*.

Keywords: Inoculation; *In ovo*; Chicken embryo; Egg; Model host; Shedding

Introduction

The genus *Cryptosporidium* comprises protozoan parasites that infect epithelial cells in the microvillus border of the gastrointestinal tract of all classes of vertebrates (Ryan and Xiao 2014). These parasites cause a self-limiting disease, frequently characterized by diarrhoea, which can become chronic and life-threatening in those who are immunocompromised. *Cryptosporidium* is a major cause of severe diarrhoea in infants and toddlers in developing countries (Striepen 2013).

To date, 43 species and more than 50 genotypes of *Cryptosporidium* have been described, of which 21 have been reported to infect humans (Čondlová et al. 2018; Ortega and Kváč 2013; Ryan and Xiao 2014). Many of these human pathogenic species and genotypes remain poorly characterized because only a few isolates are available for study. In

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contrast to other human-pathogenic protozoans, such as *Toxoplasma* (Muller and Hemphill 2013), there is a lack of in vitro methods to propagate *Cryptosporidium* oocysts (Arrowood 2002; Miller et al. 2019; Morada et al. 2016). In vivo, *C. parvum* can be propagated in several animal models (e.g. mice, piglets, calves, and gerbils) (Kváč et al. 2009; Petry et al. 1995; Vítovec and Koudela 1992), but many other human pathogenic species and genotypes have narrow host specificity and yield low numbers of oocysts in their typical hosts (Čondlová et al. 2018; Kostopoulou et al. 2015; Kváč et al. 2013; Kváč et al. 2018; Stenger et al. 2015). There is a need for more effective methods to propagate oocysts from a broad range of *Cryptosporidium* species and genotypes.

A few studies have reported the development of *Cryptosporidium* spp. in chicken embryos during prenatal host development. Current and Long (1983) showed that endoderm cells of the chorioallantoic membrane of chicken embryos supported development of *Cryptosporidium* from humans and calves, and that recovered oocysts were infectious to suckling mice. Subsequently, Lindsay et al. (1989), Wunderlin et al. (1997) and Huang et al. (2014) described the complete development of *C. baileyi* in chicken embryos when sporozoites or oocysts were inoculated into the allantoic cavity or allantoic fluid.

Cryptosporidium baileyi is the most common avian *Cryptosporidium* species (Goodwin et al. 1996) and is probably not infectious for mammals. In contrast, *C. parvum* infects various species of birds from the orders Accipitriformes, Anseriformes, Charadriiformes, Galliformes, Passeriformes, and Psittaciformes (Gomes et al. 2012; Graczyk et al. 1998; McEvoy and Giddings 2009; Nakamura et al. 2009; Reboredo-Fernandez et al. 2015; Zhou et al. 2004; Zylan et al. 2008) in addition to infecting mammals.

We hypothesized that chickens infected with *Cryptosporidium* spp. as embryos, before immune system maturation, would produce more oocysts than chickens infected post hatching. We tested our hypothesis using *C. baileyi*, a species that naturally infects chickens, and *C. parvum,* a species that is not typically infectious for chickens under experimental conditions.

Materials and Methods

Origin of animals

One-day-old ducks (*Anas platyrhynchos* f. *domestica*) and fertilized chicken eggs (*Gallus gallus* f. *domestica*) were purchased from Perena spol. s r.o., Chlumec and Cidlinou and International Poultry Testing, Ústrašice, Czech Republic, respectively. Eggs were incubated in commercially available hatcheries (Brinsea Ovation 28 EX Egg Incubator, Brinsea, Weston Super Mare, UK) at the Institute of Parasitology, Biology Centre CAS, Czech Republic. Briefly, eggs were incubated at 37.5 °C and 45% humidity for 19 days and at 37.0 °C and 65% humidity for 2 days before expected hatching. Embryos were screened for mortality on the ninth day using candling (embryo development can be seen when a bright light is shone through the eggs in a dark room), and eggs with non-developing embryos were excluded from the experiment. Immunodeficient SCID mice (*Mus musculus*) (strain C.B-17) were bred in-house at the Institute of Parasitology, Biology Centre CAS, Czech Republic.

Parasites

Oocysts of *C. baileyi* and *C. parvum* were obtained from naturally infected domestic ducks (Veselí nad Lužnicí, Czech Republic) and a calf (*Bos taurus*, Hartmanice, Czech Republic), respectively, and propagated in one-day-old ducks and eight-week-old SCID mice, respectively, at the Institute of Parasitology, Biology Centre CAS, Czech Republic. Briefly, one-day-old ducks and eight-week-old SCID mice were orally inoculated with *C. baileyi* and *C. parvum* oocysts in 200 µl distilled water, respectively, and faecal samples from the ducks and SCID mice containing oocysts of *C. baileyi* and *C. parvum* were purified using caesium chloride gradient centrifugation (Arrowood and Donaldson 1996). Purified oocysts were used to infect chicken embryos, one-day-old chickens, one-day-old ducks, and SCID mice.

The identity of *C. baileyi* and *C. parvum* isolates was verified by PCR amplification and sequencing of a fragment of the small subunit rRNA gene (18S rRNA). The *C. parvum* isolate was additionally subtyped with the 60 kDa glycoprotein gene (*gp60*).

Infection dose

Purified oocysts of *C. baileyi* and *C. parvum* were treated with sodium hypochlorite. Briefly, oocysts were centrifuged in a 1.5 ml tube at 12,000 × *g* for 3 min, the supernatant was discarded and oocysts were treated with 200 µl of sodium hypochlorite (4.7%, 4 °C, 10 min). The sodium hypochlorite was removed by washing: the 1.5 ml tube was filled with deionized water and the oocysts were washed three times by centrifugation at 12,000 × *g* for 3 min; each time the supernatant was discarded (Petry et al. 1995). After the final washing step, the oocysts were resuspended in deionized water and used for inoculation.

Experimental design

Chicken embryos were candled on the ninth day of development: unembryonated eggs were discarded, and the allantoic cavity was identified and marked. The target inoculation site on the egg shell was sanitized with 70% ethanol, punctured with a sterile, 2 mm diameter drill bit, and the infection dose was injected into the allantoic cavity. The inoculation site was sealed with sterile, liquid Histoplast-S (SERVA Electrophoresis GmbH, Heidelberg, Germany) immediately after inoculation. Groups of

embryonated chicken eggs ($n = 45$) and one-day-old chickens ($n = 45$) were inoculated with 10^4, 10^5, or 10^6 oocysts of *C. baileyi* or *C. parvum* in 50 μl deionized water. Ten embryonated chicken eggs and ten one-day-old chickens were used as negative controls. The infectivity of *C. baileyi* and *C. parvum* was verified in three ducks and three mice, respectively (positive controls). One-day-old chickens, one-day-old ducks, and eight-week-old SCID mice were inoculated orally using intragastric gavage. Eggs and chickens inoculated with a different parasite species or infection dose were housed in separate cages/hatcheries.

On each day post hatching (DPH)/infection (DPI), animals were examined for health status and screened for the presence of specific DNA and oocysts of *Cryptosporidium* in faeces using aniline-methyl-violet staining (ACMV), according to Miláček and Vítovec (1985). Preparations were viewed under 1000× magnification (Olympus BX51 microscope) and documented using Olympus cell Sens Entry 2.1 (Olympus Corporation, Shinjuku, Tokyo, Japan) and a digital camera (Olympus DP73).

Two chickens from each group were euthanized immediately after hatching and, subsequently, every third day of the experiment. Samples of organs (eye; heart; brain; kidney; liver; lung; trachea; oesophagus; ventriculus; proventriculus; proximal, medial, and distal duodenum; proximal, medial, and distal jejunoileum; caecum; colon; and cloaca) and faeces from the cloaca were collected using aseptic techniques. All organs were screened for the presence of specific DNA by PCR amplification of the 18S rRNA gene, and the developmental stage of the parasite was determined by staining of mucosal scrapings, histology sections, and scanning electron microscopy (below). Experiments were terminated at 30 DPH/DPI. If the animal developed severe disease, it was humanely sacrificed before the 30 days.

Ten chickens from each group were examined individually for *Cryptosporidium* oocysts in faeces each day for 30 DPH/DPI. The prepatent period, the timing of maximum infection intensity (T_{max}), the concentration at maximum infection intensity (C_{max}), and the patent period were monitored. Infection intensity was reported as oocyst per gram (OPG), as previously described in Kváč et al. (2007). Briefly, each animal was examined for *Cryptosporidium* oocysts using the ACMV staining method: each glass slide was weighed immediately before and after smearing (0.001 g accuracy) and stained. The entire smear was examined by light microscopy at 1000× magnification. The total number of oocysts on the slide and the weight of the faecal smear were used to calculate OPG. To estimate the daily yield of oocysts, the number of oocysts per gram was multiplied by the total weight of faeces, in grams, produced per chicken per day. Oocysts recovered from faeces of infected chickens were purified by caesium chloride gradient and examined for infectivity.

Each experiment was repeated three times and the resulting values represent the mean of all observations.

Molecular characterization

DNA was extracted from 200 mg of faeces or tissue by bead disruption for 60 s at 5.5 m/s using 0.5 mm and 2 mm glass beads in a FastPrep®24 Instrument (MP Biomedicals, CA, USA), followed by isolation/purification using a commercially available kit in accordance with the manufacturer's instructions (Exgene™ Stool DNA mini, GeneAll Biotechnology Co. Ltd, Seoul, Korea and DNeasy Blood & Tissue Kit QIAGEN, Hilden, Germany). Purified DNA was stored at −20 °C prior to being used for PCR.

A nested PCR approach was used to amplify a partial region of the 18S rRNA (~830 bp; Xiao et al. 1999) and *gp60* genes (~820 bp; Alves et al. 2003; Peng et al. 2001). For the 18S rRNA gene, primary amplification was performed with the primers 5′TTCTAGAGCTAATACATGCG3′ and 5′CCCATTTCCTTCAAACAGGA3′, followed by secondary amplification with the primers 5′GGAAGGGTTGTATTTATTAGATAAAG3′ and 5′AAGGAGTAAGGAACAACCTCCA3′. For the gp60 gene, primary amplification was performed with the primers 5′ATAGTCTTCGCTGTATTC3′ and 5′GGAAGGAACGATGTATCT3′, followed by secondary amplification with the primers 5′TCCGCTGTATTCTCAGCC3′ and 5′GCAGAGGAACCAGCATC3′. The primary PCR mixtures (30 μl reaction volume) contained: 2 μl of template DNA, 2.5 U of Taq DNA Polymerase (Dream Taq Green DNA Polymerase, Thermofisher Scientific, Waltham, MA, USA), 0.5× PCR buffer (18S rDNA) or 1× PCR buffer (*gp60*; Thermofisher Scientific), 6 mM $MgCl_2$ (18S rDNA) or 3 mM $MgCl_2$ (*gp60*), 0.1 mM each deoxynucleoside triphosphate, 100 mM each primer, and 2 μL non-acetylated bovine serum albumin (BSA; 10 mg ml^{-1}; New England Biolabs, Beverly, MA, USA). The secondary PCR mixtures were similar to those described above for the primary PCR, except that 2 μl of the primary PCR product was used as the template, the $MgCl_2$ concentration was 3 mM, and no BSA was used. The PCR programme consisted of the initial denaturation at 95 °C for 5 min, then 35 cycles of denaturation at 95 °C for 30 s, annealing temperature for 45 s, and extension at 72 °C for 30 s; the final extension lasted 10 min at 72 °C. The annealing temperature for amplification of the 18S rRNA gene was 50 °C for primary and 55 °C for secondary reaction and for the gp60 gene was 50 °C for both primary and secondary reactions. DNA of *Cryptosporidium hominis* subtype Ib and molecular grade water were used as positive and negative controls, respectively. Secondary PCR products were detected by agarose gel electrophoresis, visualized by ethidium bromide staining, and extracted using GenElute Gel Extraction Kit (Sigma, St. Louis, MO, USA). Purified secondary products were sequenced in both directions using the secondary PCR primers at a commercial company (Eurofins Genomics Germany GmbH, Ebersberg, Germany). The sequences

Table 1. Prepatent and patent period, and indicators of infection intensity in chickens inoculated as nine-day-old embryos or one-day-old hatched chickens with 10^4, 10^5 or 10^6 oocysts of *Cryptosporidium baileyi* or *Cryptosporidium parvum*. Infections were detected by microscopic (MIC) and molecular (PCR) examination of faeces from 1 to 30 days post hatching.

Parasite	Infection dose	Infection	Prepatent period		Patent period		AUC	T_{max}	C_{max}
			MIC Days (mean ± standard deviation)	PCR	MIC Days (mean ± standard deviation)	PCR	($\times 10^6$)	(day)	($\times 10^6$)
C. baileyi	10^4	Oral	5–6 (5.20 ± 0.42)		>30	>30	0.860	13	0.120
		Embryo	0*	0*	8–16 (10.80 ± 2.48)		136.264	7	12.610
	10^5	Oral	4–6 (5.40 ± 0.70)		>30	>30	0.950	12	0.120
		Embryo	0*	0*	8–15 (10.80 ± 2.52)		159.952	11	14.070
	10^6	Oral	4–5 (4.70 ± 0.78)		>30	>30	1.112	11	0.120
		Embryo	0*	0*	5–15 (10.20 ± 2.82)		139.913	9	16.620
C. parvum	10^4	Oral	ND	4–6 (5.20 ± 0.63)	ND	17–25 (21.10 ± 3.11)	ND	ND	ND
		Embryo	0*	0*	5–18 (10.30 ± 4.11)	15–24 (20.10 ± 4.23)	0.034	5	0.007
	10^5	Oral	ND	4–5 (4.60 ± 0.52)	ND	21–27 (24.10 ± 1.66)	ND	ND	ND
		Embryo	0*	0*	6–16 (10.50 ± 3.44)	13–25 (20.50 ± 4.62)	0.034	5	0.010
	10^6	Oral	ND	4–5 (4.20 ± 0.42)	ND	23–27 (24.50 ± 1.58)	ND	ND	ND
		Embryo	0*	0*	5–18 (10.80 ± 5.59)	15–25 (21.70 ± 5.01)	0.046	5	0.010

Oral per oral inoculation of one-day-old chicken; embryo inoculation of nine-day-old chicken embryo; AUC (area under curve; accumulated value of infection intensity); T_{max} maximum infection intensity time; C_{max} maximum infection intensity concentration; * animals shed oocyst immediately after hatching; # all animal died or were sacrificed due to poor health; ND not determined.

obtained in the study were compared with those in GenBank (https://www.ncbi.nlm.nih.gov/genbank/).

Histology, staining of mucosal smears, and scanning electron microscopy

The tissue specimens for histology were fixed in 10% buffered formalin and processed by the usual paraffin method. Histology sections (4 μm) were stained with haematoxylin and eosin (HE) and Periodic Acid–Schiff (PAS) stains. Giemsa and Wright staining procedures (Tyzzer 1910) were used to visualize *Cryptosporidium* spp. developmental stages scraped from mucosal surfaces. As the first step, the blood serum of chickens was obtained from non-infected controls. Tissue samples were washed with cold PBS with subsequent exposure to chicken serum for 5 min. Mucosal smears were fixed with osmium vapour for 15 min followed by Giemsa staining for 6 min. Slides for histology and mucosal smears were viewed at 200–400× and 1000× magnification, respectively, and documented using Olympus cell Sens Entry 2.1 (Olympus Corporation, Shinjuku, Tokyo, Japan) and a digital camera (Olympus DP73). Specimens for scanning electron microscopy were fixed overnight at 4 °C in 2.5% glutaraldehyde in 0.1 M phosphate buffer, washed three times for 15 min in the same buffer, post-fixed in 2% osmium tetroxide in 0.1 M phosphate buffer for 2 h at room temperature and finally washed three times for 15 min in the same buffer. After dehydration in a graded acetone series, specimens were dried using the critical point technique, coated with gold, and examined using a JEOL JSM-7401F-FE SEM.

Animal care

Chickens, ducks, and mice were kept separately in plastic cages with raised bottom grids to avoid re-infection, and supplemented with a poultry or rodent diet and sterilized water ad libitum. Additionally, the cages for chickens and ducks were heated with infra-red heat lamps for the first 7 days. Animal caretakers wore new disposable coveralls, shoe covers, and gloves every time they entered the experiment room. All wood-chip bedding, faeces and disposable protective clothing were sealed in plastic bags, removed from the experimental room, and incinerated. All of the experimental procedures were conducted in accordance with the laws of the Czech

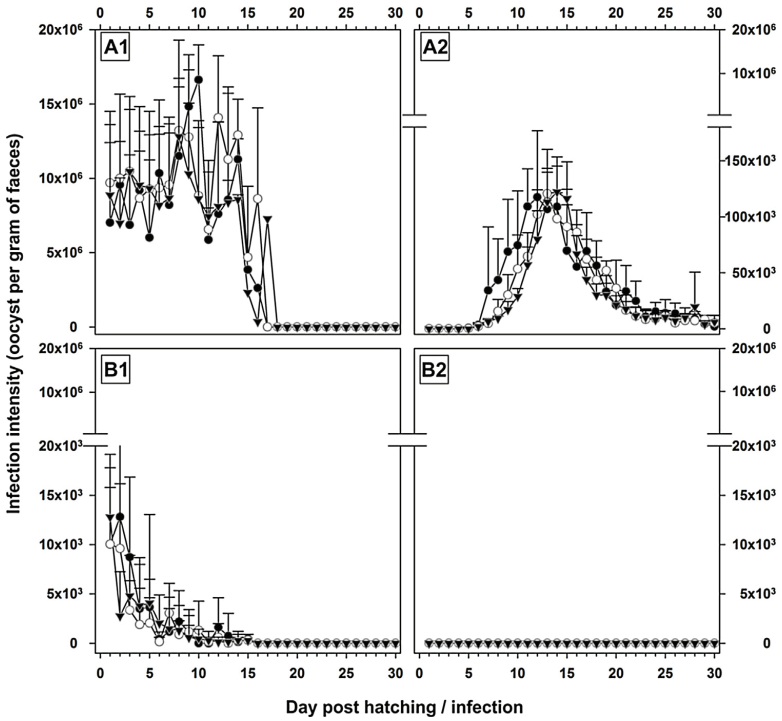

Fig. 1. Infection intensity and number of oocysts per gram of faeces (OPG) in experimentally infected chickens. (A) *Cryptosporidium baileyi* and (B) *Cryptosporidium parvum* in chickens infected with 10^4 (black triangle), 10^5 (white circle), or 10^6 (black circle) oocysts as (1) nine-day-old embryos or (2) one-day-old hatched chickens, based on molecular examination of faeces from 1 to 30 days post hatching or infection, respectively.

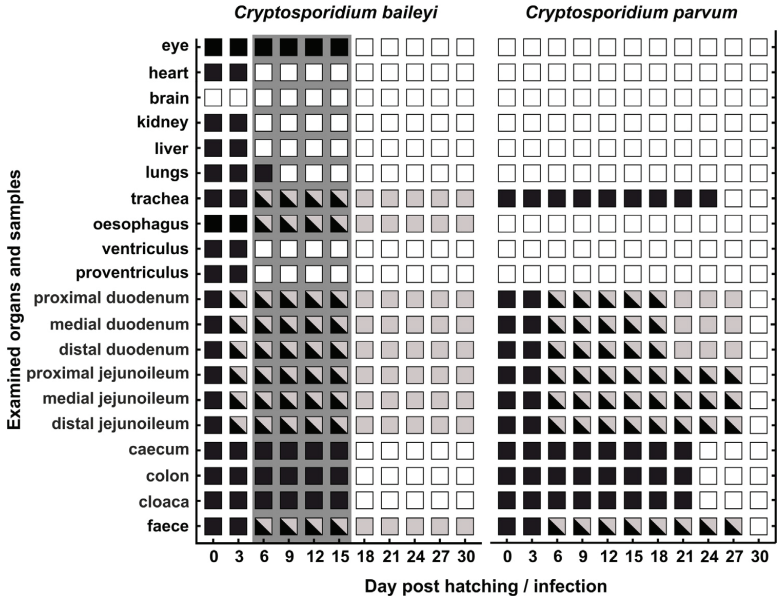

Fig. 2. Dissemination of *Cryptosporidium baileyi* and *Cryptosporidium parvum* infection to organs and tissues in chickens infected as embryos (black) and chickens infected as one-day-olds (light grey) with 10^4 oocysts, based on molecular examination from 1 to 30 days post hatching or infection, respectively. White squares indicate specific DNA was not detected. The dark grey background indicates the time when chickens infected with *C. baileyi* as embryos died or were humanely sacrificed.

Republic on the use of experimental animals, and the safety and use of pathogenic agents. The study was approved by the Institute of Parasitology, Biology Centre CAS, and Institutional and National Committees (protocol no. 60/2019).

Statistical analysis

The accumulated value of infection intensity was calculated as the area under the curve (AUC) through the classical trapezoidal rule, and T_{max}, C_{max}, and prepatent and patent period were determined. Subsequently, one-way ANOVA or a non-parametric approach through distance components (DISCO; Rizzo and Szekely 2010) in the case of nonnormality and a Dunn's post hoc test (multiple comparisons) were used. Evaluation of significance was carried out after Benjamini–Hochberg's *p*-value adjustment to control the false discovery rate. For two-sample comparisons, we used the Mann–Whitney *U* test. All computations were performed using the programming environment R 3.6.1 (R Core Team 2019).

Results

The *C. baileyi* and *C. parvum* isolates used in the study shared 100% sequence identity at the 18S rRNA locus with a *C. baileyi* isolate from a chicken [GenBank Acc. No. AF093495] and a *C. parvum* isolate from a calf [AF093493], respectively. *Cryptosporidium parvum gp60* gene sequences were subtype IIaA16G1R1, which has been reported from cattle, sheep, and humans [KJ158747, JX258866 and AM937009]. One-day-old ducks infected with *C. baileyi* and eight-week-old SCID mice infected with *C. parvum* (positive controls) began shedding oocysts after 4–5 and 10–12 DPI, respectively. 18S rRNA gene sequences of *C. baileyi* and *C. parvum* from faeces post inoculation and from the inoculum were identical.

Any difference in the course of infection among groups inoculated with the same *Cryptosporidium* sp. and same infection dose – expressed by prepatent and patent period, AUC, T_{max}, C_{max}, number of infected animals per group or amount of spreading of infection to body organs – was detected in three independent repeated experiments. All embryos and one-day-old chickens inoculated with *C. baileyi* or *C. parvum* produced microscopically and/or molecularly detectable infection.

Course of infection in chickens inoculated at the embryo stage

All chicken embryos inoculated with *C. baileyi* or *C. parvum* were hatched alive and the weight of inoculated birds did not differ significantly from uninoculated controls

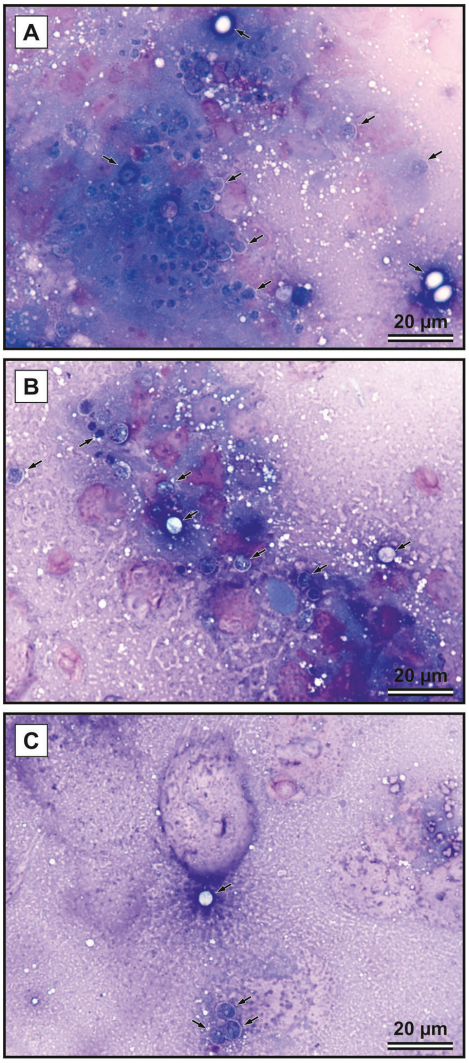

Fig. 3. Tissue smear preparations from the trachea using the Wright staining method (Tyzzer, 1910) showing the number of observed *Cryptosporidium* developmental stages. Each image shows a single field at 1000× magnification. (A) A chicken infected with *Cryptosporidium baileyi* as an embryo; (B) a chicken infected with *Cryptosporidium baileyi* at one day old; (C) a chicken infected with *Cryptosporidium parvum* as an embryo. All animals were infected with 10^4 oocysts and sacrificed six days post hatching (infected as embryos) or infection (infected as one-day-olds). Scale bar included in each picture.

($F = 0.4806$, p-value = 0.6236; data not shown). One chicken that hatched from an embryo inoculated with 10^6 *C. baileyi* oocysts was humanely sacrificed five DPH due to low weight and poor health. Microscopic and molecular analyses showed the presence of oocysts and specific DNA in faecal samples of all inoculated birds within 5 h of hatching, with no difference in infection intensity among groups receiving different infection doses ($F_{disco} = 1.051$, p-value = 0.382; Fig. 1). While chickens inoculated with *C. baileyi* shed detectable oocysts every day during the experiment, chickens shed *C. parvum* oocysts intermittently (Fig. 1). However, *C. parvum*-specific DNA was detected almost daily up to 27 DPH (Fig. 1). All chickens inoculated with *C. parvum* cleared the infection within 13–25 days and survived until the end of the experiment (Table 1). The health status of *C. parvum* inoculated chickens did not differ from non-inoculated controls.

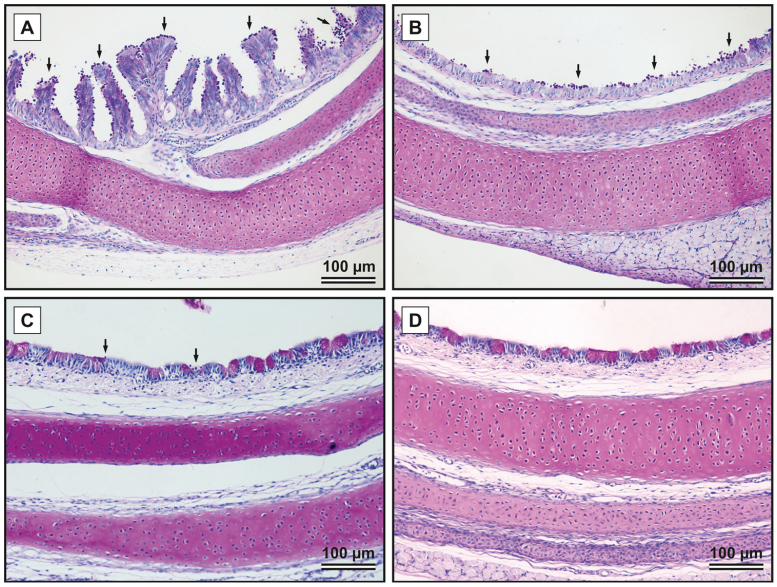

Fig. 4. Histology sections of the trachea stained with Periodic Acid–Schiff showing the presence of *Cryptosporidium* developmental stages on the epithelium (arrows). Each image shows a single field at 200× magnification. (A) A chicken infected with *Cryptosporidium baileyi* as an embryo; (B) a chicken infected with *Cryptosporidium baileyi* at one day old; (C) a chicken infected with *Cryptosporidium parvum* as an embryo; (D) a chicken infected with *Cryptosporidium parvum* at one day old. All animals were infected with 10^4 oocysts and sacrificed six days post hatching (infected as embryos) or infection (infected as one-day-olds). Scale bar included in each picture.

In contrast, all chickens infected with *C. baileyi* died or were humanely sacrificed due to poor health within 5–16 DPH. All chickens infected with *C. baileyi* as embryos were anorexic, showed a loss of appetite and decreased activity, and had insufficient feathering. Additionally, 48 h before death, there was an accumulation of faeces on the outside of the cloaca that made it difficult for the birds to defecate.

In chickens infected with *C. baileyi*, the highest oocyst production, measured as AUC, was observed in birds inoculated with 10^5 oocysts, but the AUC did not differ significantly among groups receiving different infection doses ($F_{disco} = 0.448$, p-value = 0.896). Similarly, infection dose had no effect on the AUC in chickens infected with *C. parvum* ($F = 2.3422$, p-value = 0.1153; Table 1). The highest concentration at maximum infection intensity (C_{max}) was observed in chickens inoculated with 10^6 oocysts, but differences among groups were not statistically significant ($F_{disco} = 1.622$, p-value = 0.1049 for *C. baileyi*; $F_{disco} = 1.819$, p-value = 0.1009 for *C. parvum*). The timing of maximum infection intensity (T_{max}) did not correlate with infection dose ($F_{disco} = 1.051$, p-value = 0.382 for *C. baileyi*; $F_{disco} = 0.975$; p-value = 0.40859 for *C. parvum*; Table 1).

There was no difference in the survival of chickens inoculated with different infection doses of *C. baileyi* ($F = 0.052$, p-value = 0.9948) or *C. parvum* ($F_{disco} = 0.564$, p-value = 0.7043).

Course of infection in chickens inoculated at one-day old

Microscopic and molecular analyses showed the presence of oocysts and/or specific DNA in faecal samples from all inoculated animals after the prepatent period (Table 1 and Fig. 1). Inoculated one-day-old chickens started to shed oocysts of *C. baileyi* at 4 to 6 DPI. Chickens inoculated with 10^6 oocysts of *C. baileyi* began shedding oocysts significantly earlier than those inoculated with lower doses ($F_{disco} = 3.296$, p-value = 0.013986; Table 1.). Chickens inoculated with *C. parvum* did not shed microscopically detectable oocysts, but *C. parvum*-specific DNA was detected in faeces, and it was detected significantly earlier in chickens inoculated with 10^6 oocysts than in chickens inoculated with lower doses ($F_{disco} = 3.956$, p-value = 0.008991; Table 1 and Fig. 1). In contrast to chickens inoculated with *C. baileyi* as embryos, which all died, all the chickens inoculated as one-day-olds survived. The highest oocyst production (AUC) and the earliest achievement of maximum production (T_{max}) was observed in chickens inoculated with 10^6 oocysts of *C. baileyi* ($F = 3.7899$, p-value = 0.03542 for AUC; and $F_{disco} = 3.721$, p-value = 0.006993 for T_{max}; Table 1).

While the patent period did not differ among birds inoculated with different infection doses of *C. baileyi* ($F_{disco} = 0.265$, p-value = 0.51249), birds inoculated with 10^4

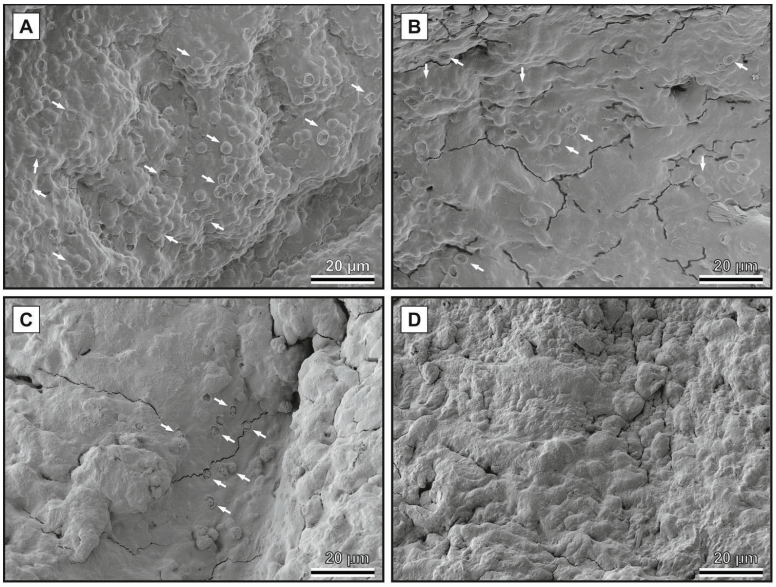

Fig. 5. Scanning electron photomicrographs of a trachea showing the presence of *Cryptosporidium* developmental stages on the epithelium (arrows). Each image shows a single field at 10,000× magnification. (A) A chicken infected with *Cryptosporidium baileyi* as an embryo; (B) a chicken infected with *Cryptosporidium baileyi* at one day old; (C) a chicken infected with *Cryptosporidium parvum* as an embryo; (D) a chicken infected with *Cryptosporidium parvum* at one day old. All animals were infected with 10^4 oocysts and sacrificed six days post hatching (infected as embryos) or infection (infected as one-day-olds). Scale bar included in each picture.

oocysts of *C. parvum*, the lowest infection dose tested, cleared the infection earlier than birds inoculated with higher infection doses ($F_{disco} = 3.270$, p-value = 0.024975).

Spreading of *Cryptosporidium* spp. infection to body organs

In all chickens that were inoculated with *C. baileyi* as embryos, specific *Cryptosporidium* DNA was detected in all organs except the brain immediately after hatching. The infection was cleared from the liver, kidney, proventriculus, and ventriculus by day six post hatching. All other organs remained positive until the animals died or were sacrificed. The number of organs infected did not differ among birds inoculated as embryos with different infection doses. In contrast, only the trachea, oesophagus, duodenum, and jejunoileum were positive in chickens inoculated with *C. baileyi* as one-day-olds (Fig. 2). Chickens inoculated with *C. parvum* as one-day-olds only developed infections in the small intestine, regardless of infection dose. In contrast, chickens infected with *C. parvum* as embryos additionally developed infection in the trachea, cecum, colon, and cloaca (Fig. 2). Specific DNA of *C. baileyi* was detected in the proventriculus, ventriculus, liver, kidney, lung, heart, and eye in chickens inoculated as embryos, but developmental stages were not detected in any of these organs. In birds inoculated with *C. baileyi* as embryos or as one-day-olds, developmental stages were detected in mucosal smears from the trachea, oesophagus, and gut (Figs. 3–5). While *C. parvum* developmental stages were occasionally observed in mucosal smears of the trachea and gut of chickens infected as embryos, they were not detected in birds infected as one-day-olds (Figs. 3–5).

Oocyst production efficiency

Generally, chickens inoculated with *C. baileyi* (p-value = 1.122×10^{12}) and *C. parvum* (p-value = 0.02785) at one day old shed significantly fewer oocysts than chickens inoculated as embryos (Table 1; Figs. 6 and 7). Chickens inoculated as embryos with 10^4 oocysts of *C. baileyi* produced approximately 2.8×10^9 oocysts during the first 17 DPH, which is ~85 times greater than the oocyst production by chickens infected as one-day-olds (3.0×10^7 oocysts during the first 21 DPI; Fig. 7). The ratio between the inoculum dose and oocyst yield from chickens inoculated as embryos and chickens inoculated as one-day-olds was 1:280,000 and 1:3200, respectively. The greatest daily output of *C. baileyi* oocysts in chickens inoculated as embryos was observed at eight DPH (Fig. 7). While chickens infected as one-day-olds with *C. parvum* did not produce detectable oocysts, chickens inoculated as embryos with 10^4 oocysts of *C. parvum* pro-

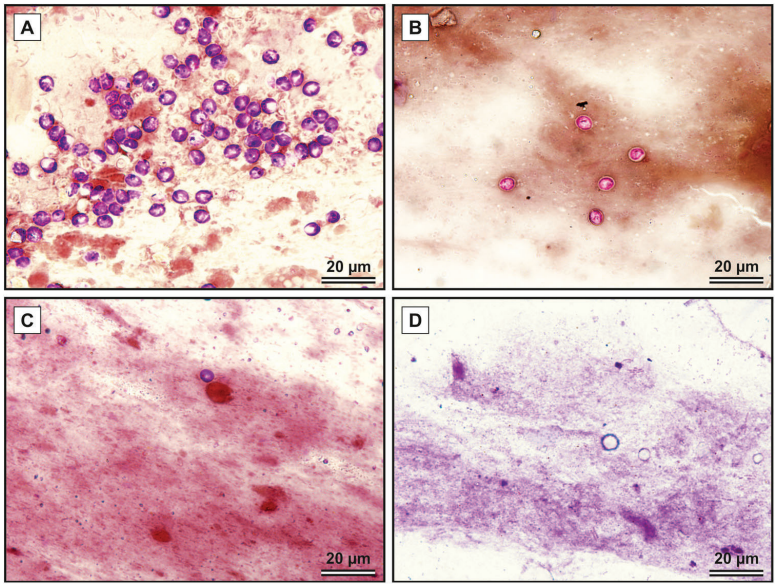

Fig. 6. Faecal smears stained with aniline-carbol-methyl-violet showing the intensity of oocyst shedding. Each image shows a single field at 1000× magnification. (A) A chicken infected with *Cryptosporidium baileyi* as an embryo; (B) a chicken infected with *Cryptosporidium baileyi* at one day old; (C) a chicken infected with *Cryptosporidium parvum* as an embryo; (D) a chicken infected with *Cryptosporidium parvum* at one day old (no oocysts were found). All animals were infected with 10^4 oocysts and sacrificed six days post hatching (infected as embryos) or infection (infected as one-day-olds). Scale bar included in each picture.

duced approximately 4.0×10^5 oocysts during the first 15 DPH. The ratio between the inoculum dose and oocyst yield was 1:35. The greatest daily output of *C. parvum* oocysts in chickens inoculated as embryos was observed at five DPH (Fig. 7).

Discussion

A major barrier to experimental studies of many *Cryptosporidium* species and genotypes is the lack of methods to propagate oocysts. In previous work (Current and Long 1983; Lindsay et al. 1989), *C. baileyi* from a bird and *Cryptosporidium* spp., probably *C. parvum*, isolated from a human and a calf were found to develop in the chorioallantoic membrane of chicken embryos following experimental inoculation. Post-hatching development was not examined. The presence of *C. parvum*-specific DNA has been reported in various bird species (Ferrari et al. 2018; Gomes et al. 2012; Helmy et al. 2017; Ng et al. 2006; Oliveira et al. 2017; Santín et al. 2004), but it is unclear whether birds are naturally infected or are mechanical vectors (Plutzer and Tomor 2009; Quah et al. 2011). Graczyk et al. (1998) detected *C. parvum* shedding at least nine DPI in five experimentally infected Canada geese (*Branta canademis*), but concluded that the shedding may have been due to inoculum passage. The recovery of infectious, mature *C. parvum* oocysts from an asymptomatic rhea (*Rhea americana*) could be considered an ongoing infection (Krindges et al. 2013). The present study has shown that chickens infected with *C. parvum* as embryos produce 35 times more oocysts than are present in the inoculum, demonstrating that *C. parvum* is being propagated. In contrast, chickens infected at one day old did not produce oocysts detectable by microscopy. In comparison with a calf, which can potentially produce 10^{10} *C. parvum* oocysts (Nydam et al. 2001), a chicken infected at the embryo stage produces only 3×10^5 oocysts; however, the level of care and cost involved in the chicken model is far lower than that required for a calf.

The production of *C. baileyi* oocysts by chickens infected as one-day-olds was expected and is in accordance with previous reports (Abbassi et al. 1999; Lindsay and Blagburn 1990). Similarly, the observed gradual loss of *C. baileyi* infection in chickens infected at one day old has been previously reported (Sreter and Varga 2000; Sreter et al. 1995). Here, we have additionally shown that the chickens infected as embryos develop intense infections, with a massive oocyst output that amplifies the inoculum 280,000 times. The total oocyst output from chickens infected as embryos was about 8000 times greater than from chickens infected as one-day-olds. While we do not know how much oocyst amplification occurred in the egg, pre-hatching, Wunderlin et al. (1997) found that eggs produced only about half the number of *C.*

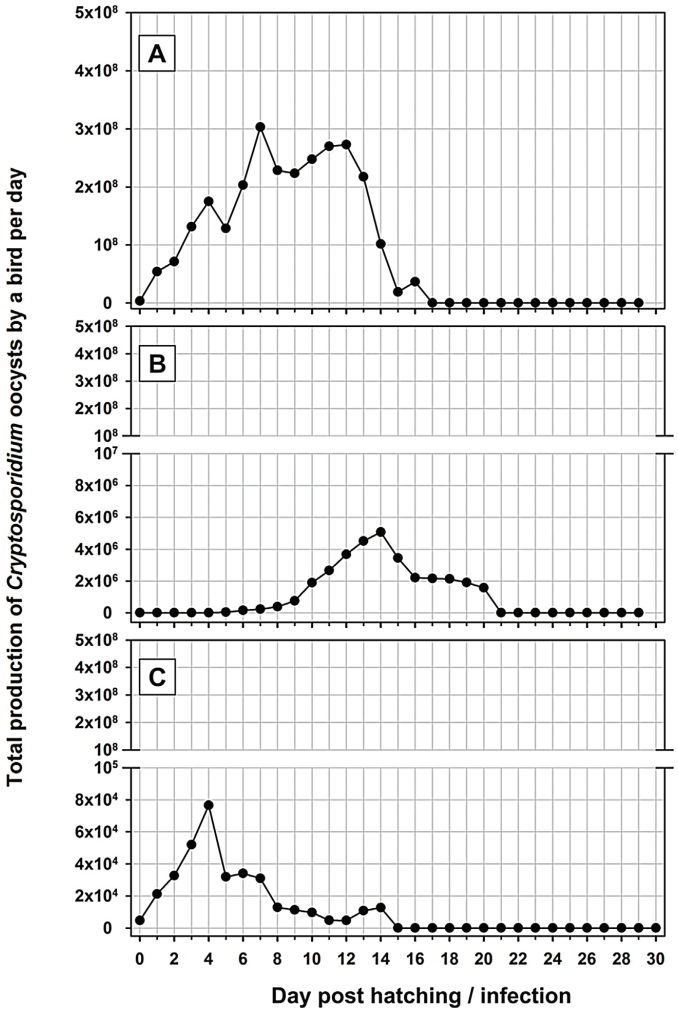

Fig. 7. The approximate total production of *Cryptosporidium* oocysts per bird, calculated from the daily AUC and total daily faecal production in chickens infected with (A) *Cryptosporidium baileyi* as embryos, (B) *Cryptosporidium parvum* as embryos, and (C) *Cryptosporidium baileyi* as one-day-olds. All animals were infected with a 10^4 oocysts.

baileyi oocysts as chickens that were infected at two days old. Therefore, the massive amplification of oocysts requires inoculation at the embryo stage and recovery of oocysts from hatched chickens.

Our finding that the course of infection was not affected by infection dose is consistent with previous reports (Kváč et al. 2016; Yousof et al. 2017; Zambriski et al. 2013) and suggests that this method of propagation can be successful even when few oocysts are available for inoculation. This is important because many natural hosts of *Cryptosporidium* shed low numbers of oocysts.

In agreement with previous studies, *C. parvum* and *C. baileyi* infection in one-day-old chickens was not associated with clinical signs of disease (Ferrari et al. 2018; Gomes et al. 2012; Graczyk et al. 1998; Hatkin et al. 1990; Helmy et al. 2017; Ng et al. 2006; Oliveira et al. 2017; Santín et al. 2004). In contrast, 100% mortality occurred within 15 days of hatching in chickens infected with *C. baileyi* as embryos.

As observed in mammals and other avians, where *C. parvum* develops primarily in the small intestine, developmental stages were detected only in the duodenum and jejunoileum of chickens infected as one-day-olds (Li et al. 2013; Tzipori et al. 1983; Vítovec and Koudela 1992; Zylan

et al. 2008). However, chickens infected as embryos additionally had developmental stages in the caecum, colon, and trachea. Previous reports of *C. parvum* infection in the large intestine have been in hosts with an immunodeficiency (Certad et al. 2007) or carcinoma (Osman et al. 2017). The development of *C. parvum* in the trachea is consistent with studies by Azmanis et al. (2018) and Palkovič and Maroušek (1988), who described the presence of this species in the trachea of naturally and experimentally infected birds, respectively.

Cryptosporidium baileyi has broad tissue specificity in naturally and experimentally infected birds, with reports of developmental stages in the conjunctiva, nasopharynx, trachea, bronchi, air sacs, small intestine, large intestine, ceca, cloaca, bursa of Fabricius, kidneys, and urinary tract (Abbassi et al. 1999; Hatkin et al. 1990; Lindsay and Blagburn 1990; Ryan and Xiao 2008). However, similar to our study, developmental stages are mostly limited to the gut, bursa of Fabricius, and cloaca following oral infections (Blagburn et al. 1991; Goodwin and Brown 1989; Chvala et al. 2006; Lindsay et al. 1987; Surl et al. 2003).

The susceptibility of chickens infected as embryos to more intense, multi-organ infections is probably due to the immaturity of their immune systems. Lindsay et al. (1988), Taylor et al. (1994), and Sreter et al. (1995) found that innate resistance to *C. baileyi* is age related: older birds with better-functioning immune systems are able to clear the infection earlier and shed lower numbers of oocysts. The course of *C. baileyi* infection in chickens infected as embryos has parallels with congenital toxoplasmosis, where infection spreads throughout the body, subclinical disease occurs in the most affected individuals and, if untreated, infection is associated with severe and even fatal disease (McAuley 2014).

Conclusions

This study has shown that chickens hatched from infected embryos produce 280,000 times more *C. baileyi* and 35 times more *C. parvum* oocysts than the infection dose, suggesting that this could be an alternative, cost-effective model for the propagation and study of *Cryptosporidium* spp. To determine the broad applicability of this chicken embryo model for oocyst propagation, future studies should examine the infectivity of other *Cryptosporidium* spp.

CRediT author statement

Nikola Holubová: Supervision, validation, investigation, conceptualization, writing – original draft. **Bohumil Sak:** Resources, investigation, supervision. **Tereza Schulzová:** Resources, investigation. **Roman Konečný:** Formal analysis, investigation. **Rost Michael:** Software, formal analysis. **Lenka Tůmová:** Investigation, resources. **John McEvoy:** Conceptualization, visualization, writing – original draft, writing – review & editing. **Martin Kváč:** Funding acquisition, project administration, conceptualization, validation, writing – original draft, writing – review & editing.

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