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Ecological and public health significance of *Enterocytozoon bieneusi*

Wei Li [a,*], Lihua Xiao [b,*]

[a] Heilongjiang Key Laboratory for Zoonosis, College of Veterinary Medicine, Northeast Agricultural University, Harbin, Heilongjiang, China
[b] Center for Emerging and Zoonotic Diseases, College of Veterinary Medicine, South China Agricultural University, Guangzhou, Guangdong, China

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ABSTRACT

Enterocytozoon bieneusi, a fungus-like protist parasite, causes symptomatic and asymptomatic intestinal infections in terrestrial animals and is also abundant in the environment. This parasite has been isolated from a variety of host types including humans, livestock, companion animals, birds, and wildlife, as well as the natural and urban environments including drinking source water, coastal water, recreational water, wastewater, vegetables in retail markets, and raw milk on farms. *E. bieneusi* exhibits high genetic diversity among host species and environmental sources and at least 500 genotypes have been identified thus far. Since its discovery in AIDS patients in 1985, scientists across the world have worked to demonstrate the natural history and public health potential of this pathogen. Here we review molecular typing studies on *E. bieneusi* and summarize relevant data to identify the potential sources of human and nonhuman infections and environmental contamination. This review also discusses the possible transmission routes of *E. bieneusi* and the associated risk factors, and advocates the importance of the One Health approach to tackle *E. bieneusi* infections.

1. Introduction

In the broadest sense, the science of ecology can be defined as the study of the interactions between organisms and their environment. Microbial ecology examines the interactions between microorganisms, their hosts, and the environment. Publications on the abundance, distribution, and genetic variation of *Enterocytozoon bieneusi* among hosts in the context of host specificity and zoonotic potential have been reviewed [1–6], whereas there has been no review interpreting the ecological aspects relating to the environmental transport of this pathogen in nature and the associated public health implications.

Microsporidia comprises a highly diverse group of obligate intracellular parasites colonizing an extremely wide range of eukaryotes [7–9]. Over 200 microsporidian genera and nearly 1500 species have now been described [3], of which the most frequently reported in human microsporidiosis is *E. bieneusi* [10]. The life cycle of *E. bieneusi* involves two major stages, an infective or environmental spore stage and a disease-causing vegetative stage. Spores are ingested by the susceptible hosts, invade the epithelial cells lining the gut lumen, and replicate intracellularly. Fresh spores are released into the environment via feces [3]. The rigid three-layered spore wall may play a vital role in the survival of the parasite in hostile environments [11,12]. In line with the importance of environmental contamination in its transmission, *E. bieneusi* has caused food- and waterborne and hospital-related outbreaks [13–16]. Most cases of microsporidian infections in HIV-infected persons are attributable to *E. bieneusi* and the infections manifest as varying clinical symptoms, typically chronic diarrhea and wasting [7]. While oral fumagillin has been considered as a possible method for eradicating this opportunistic pathogen from the intestinal tract of AIDS patients, it is highly toxic and not commercially available in most countries [17,18]. As a consequence, a promising therapy for *E. bieneusi*-associated microsporidiosis remains to be explored. In addition, it is very difficult to expand anti-*E. bieneusi* treatment options due to the limitation of in vitro propagation of the parasite [19]. As there are repeated reports of the parasite in asymptomatic immunocompetent individuals, notably children and the elderly, it may be argued that the association between *E. bieneusi* infection and diarrhea is not as tight as it was initially believed to be [10].

Specific diagnosis of *E. bieneusi* by conventional staining methods is difficult to achieve due to the extremely small size of the spore (around 1 μm) and the interference from food particles, bacteria, fungi, and other microsporidian species like *Encephalitozoon* spp.; transmission electron microscopy is a powerful means for diagnosis, but is not available in most settings [7,12,20]. Immunodetection with monoclonal antibodies is readily applicable to large-scale epidemiological surveys, but no genotyping information can be provided [21–23]. With the advent of

* Corresponding authors.
E-mail addresses: neaulw@gmail.com (W. Li), lxiao@scau.edu.cn (L. Xiao).

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molecular techniques, such as PCR and DNA sequencing of the ribosomal internal transcribed spacer (ITS), *E. bieneusi* has been characterized worldwide from humans, a great variety of domestic and wild animals (pigs, monkeys, cattle, deer, sheep, goats, dogs, cats, horses, carnivores, rodents, birds, etc.), and water environment [3,4], arousing our curiosity about the sources of infection and modes of transmission. Current literature lacks consensus on the definition of the natural reservoir hosts of *E. bieneusi* since the parasite can infect multiple animal species, rather than specific target populations. Asymptomatic *E. bieneusi* infections are quite common in animals [4]. There is seemingly no consistent correction between *E. bieneusi* occurrence and animal diarrhea [24–26]. Human *E. bieneusi* infections may be acquired by contact-associated zoonotic transmission or by ingestion of spores from environmental sources [10,27]. Many cases are likely resulted from human to human transmission [28,29].

Water contamination with pathogenic microorganisms is one of the serious threats to human and animal health, and is also an important issue of environmental and ecological concerns all over the world. Major sources of microbial contamination in water include feces from humans, livestock, poultry, and wild animals through surface water runoff, wastewater discharge, and agricultural effluents [30]. Intestinal carriage of *E. bieneusi* is ubiquitous in mammalian and avian hosts [3], and via fecal matter from those hosts, environments (mainly water) can readily become contaminated [31–35]. ITS genotyping potentially allows the discrimination of environmental *E. bieneusi* isolates, helping to identify the host source of environmental contamination as well as improving our knowledge of the routes of cross-species or zoonotic *E. bieneusi* transmission [36–38]. In addition, concerns have been raised regarding the importance of fresh produce contaminated with *E. bieneusi* spores in foodborne microsporidiosis [39].

Despite the abundance, richness, diversity, and zoonotic potential of *E. bieneusi*, the ecological aspects of this parasite remain largely unknown. This creates serious difficulties in elucidating the natural history and mode of transmission of *E. bieneusi* and catching the inherent link between the environment and the health of humans and animals. All these strongly urge on us the need for applying a One Health approach to tackle *E. bieneusi* infections.

2. Molecular typing and phylogenetic groups of *E. bieneusi*

Polymorphism analysis of the ITS region of the rRNA gene and phylogenetic assessment allowed the identification of at least 500 *E. bieneusi* genotypes distributed in 11 phylogenetic groups [2,3]. Three sets of nested PCR primers that cover the entire ITS region have been broadly applied in genotyping studies [2,40–43]. *E. bieneusi* genotypes in different groups display varying degree of host specificity and zoonotic potential [3]. Group 1 is the largest group that comprises over 300 genotypes isolated from almost all current known host types, some (e.g., D, EbpC, and Type IV) of which exhibit remarkable adaption to life within a diverse array of host and natural environments [2,3]. By contrast, some Group 1 genotypes show strong host specificity, for instance, genotypes B and C are thus far confined to human infections in multiple geographic areas, and EbpB to pig infections likewise [2,3]. Only minor ITS sequence divergence (about 20 single nucleotide substitutions) is present between *E. bieneusi* genotypes from Group 1 and Group 2. Group 2 is the second largest cluster previously considered adapted to ruminants; most of its members are the most frequent contributors to *E. bieneusi* infections in cattle, deer, sheep, and goats, including BEB4, BEB6, I, and J [3,5]. These four successful genotypes probably have experienced host range expansion, leading to their human infectivity [3]. In recent years, there has been a rapid increase in the number of Group 1 and Group 2 genotypes and the host range of some of them has been extended [3]. *E. bieneusi* genotypes in Groups 3 to 11 are genetically divergent from those in Groups 1 and 2; the former mostly have a restricted host range and thus represent a minor or unknown public health threat [3]. Currently, there are many reports of genetic variants of the existing *E. bieneusi* genotypes as new ones; some of these could be due to sequencing errors, lack of proofreading of nucleotide differences in the ITS region of the PCR products, and improper consideration of polymorphisms in the flanking regions (small and large subunits) of the ITS [3,4,6,44]. In addition, caution should be taken when discussing cross-species transmission potential of *E. bieneusi* genotypes among humans, animals, and birds, as clinical, genetic, epidemiological, or ecological factors could also affect the outcome. After all, genetic identity or relatedness in the ~243-bp ITS region might not reflect the overall genetic characteristics of the *E. bieneusi* genome (~6 megabases) [45,46].

Multilocus sequence typing (MLST) has been shown to be more discriminatory since it considers genetic polymorphisms of four mini- and microsatellites in addition to the ITS [47]. MLST-based population genetic analysis by evaluating the intragenic and intergenic linkage disequilibrium (LD), standardized index of association, neutrality, and recombination events has revealed the widespread occurrence of clonality among *E. bieneusi* populations from various host species and geographical regions [48–52]. Population subdivision was determined by both multilocus phylogenetic analysis and substructural analysis; the estimation of Wright's fixation index and gene flow was performed to assess the level of genetic differentiation between subpopulations [48–52]. Further genetic analysis and host range analysis have indicated that *E. bieneusi* subpopulations might have different genetic structures (clonal/epidemic), levels of host specificity (low/strong), and transmission pathways (interspecies/intraspecies) [1,2]. Genetic network analysis has demonstrated that *E. bieneusi* isolates more often evolve from low to strong host specificity; the LD decay and relatively high genetic diversity in the subpopulations with a clonal structure might be consequences of broad host range and adaptation to new host environments [48]. Such findings have implications for the application of the current MLST scheme as a One Health typing tool for ecological surveillance of *E. bieneusi*. One limitation of the current MLST tool is its poor amplification efficiency in the analysis of the isolates from Groups 2 to 11 [2]. Based on the existing MLST evidence, whilst limited, it can be speculated that there might be other species in the genus *Enterocytozoon* [1]. Additional genetic markers are desirable for delimiting *Enterocytozoon* species [6].

3. Reservoir hosts of human-pathogenic *E. bieneusi*

The usual vehicles of *E. bieneusi* dispersal include both domestic and wild animals (Fig. 1), and among host species the pathogen displays genetic and phenotypic diversity [4,5]. Humans are infected predominately by Group 1 genotypes (e.g., D, EbpC, Type IV, A, and Peru8), and very occasionally by the genotypes from other genetic groups (e.g., Group 2 genotypes I, J, BEB4, and BEB6, Group 5 genotype KIN-3, and Group 6 genotypes MAY1, Nig3, and Nig4) [3]. There seems no geographic segregation among human *E. bieneusi* isolates as inferred from either ITS or MLST phylogeny [50,53]. Nonhuman primates (NHPs) are well-known carriers of human-pathogenic *E. bieneusi* genotypes, such as D, Type IV, EbpC, EbpA, Peru8, and Peru11, and they can also harbor some potentially NHP-specific genotypes like KB-5, KB-6, and Gorilla 3 [3]. There appears to be a high possibility of cross-species transmission of *E. bieneusi* between humans and laboratory NHPs since the latter can be the host of some common human-pathogenic genotypes such as D, Type IV, Peru8, and Peru11 [54–56]. There is seemingly no barrier to *E. bieneusi* transmission among free-ranging park/zoo NHPs and other animals nearby, because they are often in close contact with each other and the NHPs can spread generalist genotypes D, EbpC, and Type IV [57–62]. Wild NHPs that often carry genotype D are also of environmental and public health interest, since in many cases they serve as habitual frequenters of rural or suburban areas and interact directly with villagers and grazing domestic animals, potentially initiating cross-species transmission of *E. bieneusi* [63–67].

Pigs. Pigs have a high carriage rate of *E. bieneusi* and the great

Fig. 1. Schematic diagram showing the ecological and public health significance of *Enterocytozoon bieneusi* and the major routes of transmission.

majority of genotypes identified belong to Group 1, with zoonotic genotypes EbpC, EbpA, O, H, and D being the most frequent and abundant. Therefore, pigs are considered major reservoir hosts of human-pathogenic *E. bieneusi* [3]. The zoonotic nature of some genotype D/EbpC isolates was already supported by MLST analysis [1]. The first documented case of pig *E. bieneusi* infection occurred on a farm in 1996 in Switzerland and the disease persisted for months [68]. Experimental infection of gnotobiotic piglets with *E. bieneusi* spores derived from humans, macaques, and infected pigs provided convincing evidence for cross-species transmission potential [42,69]. Substantial molecular epidemiological studies on a global scale highlighted the role of farmed pigs in the dispersal of *E. bieneusi* genotypes of public health importance, notably EbpC [24,26,42,70–75], probably through animal waste slurry from intensive piggeries, which facilitates contamination of surface water and vegetables, thereby promoting waterborne and foodborne transmission of the pathogen (Fig. 1). Indeed, it has been suggested that persons who come into contact with farmed and household-raised pigs may face high risk of *E. bieneusi* infection [75–77]. Free-roaming feral pigs may also play a role in *E. bieneusi* dispersal since they usually shuttle between human and animal hosts and between home and natural environments (Fig. 1). Wild boars and farmed pigs have similar distribution pattern of *E. bieneusi* genotypes [78,79]. It has become evident that genotypes EbpA and PigSpEb1 might be transmitted between sympatric extensively farmed pigs and free-ranging wild boars [80].

Ruminants. Cattle, sheep, goats, and deer are common reservoir hosts of *E. bieneusi* [3]. Phenotypic and genotypic profiles of *E. bieneusi* isolates from ruminants show marked heterogeneity comparable with those from pigs; ruminants are major hosts of Group 2 genotypes, such as BEB4, BEB6, I, and J, and they can also carry some Group 1 genotypes such as EbpC, D, Type IV, and Peru6, and several divergent genotypes (CM4 and S7) in other genetic groups [3]. Frequent interactions among farmed ruminants (cattle, sheep, and goats) may facilitate the transmission of some *E. bieneusi* genotypes; animal wastes from feedlots can conceivably spread some genotypes (e.g., BEB4, BEB6, I, J, EbpC, D, and Type IV) with broad host ranges to the residents and uninfected animals, and contaminate vegetable field and water supplies [81–88]. Milk contaminated with genotypes D, Type IV, I, and J can also act as an infection source of human microsporidiosis [89]. Due caution should be exercised to the potential cross-species transmission of generalist genotypes EbpC, D, Type IV, BEB4, BEB6, I, and J between free-ranging domestic ruminants and other domestic and wild animals that share the same outdoor or grazing areas (Fig. 1). In addition, we should not discount domestic and wild deer as potential reservoirs for some human-infective genotypes such as D, EbpA, EbpC, Type IV, Peru6, BEB6, I, and J [90–93], as well as farmed takins and zoo-bred alpacas for genotypes D, BEB6, I, and J [61,83,94].

Companion animals. Cats and dogs living as human companions and free-roaming animals can potentiate zoonotic or cross-species transmission of *E. bieneusi*. Cats are the common carriers of the most common human-pathogenic genotypes D and Type IV. In contrast, dogs around the world mostly harbor a host-adapted genotype PtEb IX (a Group 11 genotype), although they can also host some human-pathogenic genotypes such as D, EbpC, O, and A [95–105]. There appears to be no significant differences in genotype distribution between household and stray cats/dogs [3]. Companion animals might be the most direct source of *E. bieneusi* contamination in the home environment, and in turn can acquire infections through contacts with fecal matters of humans and other household animals (Fig. 1). Stray cats and dogs are at increased risk of exposure to *E. bieneusi* infections from various sources including spore-containing human and animal wastes, food, and water, thus facilitating the dispersal of *E. bieneusi* in the environment.

Birds. Close contacts with birds can also transmit *E. bieneusi* infections [3]. Similar to livestock, fecal materials from farmed chickens, geese, ducks, pigeons, falcons, and ostriches and the organic wastes from these farms can also be important contributors to *E. bieneusi* in the environment (Fig. 1), due to the common occurrence of zoonotic genotypes D, Type IV, BEB6, J, Peru6, Peru11, and Henan-IV in those birds [72,106–111]. Humans and household animals in theory can acquire *E. bieneusi* infections by genotypes D, EbpC, A, EbpA, and Peru6 from exotic birds, although direct evidence for this type of cross-species transmission is lacking [83,112–115]. Urban pigeons and rooks and wild migratory cranes can act as reservoirs of genotypes D, J, EbpA, and Peru6 [109,112,114,116–121]; through fecal droppings of those free-flying birds, human settlement environments, livestock farms and grazing areas, wildlife habitats, livestock/wildlife interface areas, farmlands, and surface water may become contaminated with *E. bieneusi* spores (Fig. 1).

Other domestic, captive/exotic, and wild animals. Those animals such as equines, carnivores, rodents, and lagomorphs are also commonly infected with *E. bieneusi*, thus have the potential for disseminating *E. bieneusi* spores. The role of such reservoir hosts, particularly free-roaming wild rodents, in the environmental transport and ecology of human-pathogenic *E. bieneusi*, cannot be ignored because of their carriages of genotypes D, EbpC, Type IV, Peru6, I, and J [3]. A number of epidemiologic investigations have assessed the public health potential of *E. bieneusi* infections from farmed/grazing horses and donkeys, farmed/household-raised rabbits, and farmed/zoo-bred foxes, raccoon dogs, raccoons, lions, tigers, bears, pandas, meerkats, and kangaroos [61,72,83,122–129]. A previous report showed the possible transmission of a unique genotype Peru16 between children and guinea pigs that live in the same household [27]. As shown in Fig. 1, *E. bieneusi* may be maintained in a natural transmission cycle involving wild free-living mammals, such as foxes, raccoons, bears, otters, beavers, squirrels, chipmunks, woodchucks, muskrats, voles, mice, and cottontails, since they can carry zoonotic genotypes D, EbpC, Peru11, or Type IV [37,43,72,130,131]. *E. bieneusi* spores in feces of wild animal reservoirs can contaminate source water via storm runoff [37,43]. The free-ranging wildlife, notably small rodents, may maintain the cycling of *E. bieneusi* among humans, livestock, wildlife, and the environment (Fig. 1) [130,131].

In spite of widespread presence of *E. bieneusi* in terrestrial hosts and the important ecological roles of those hosts in dispersing spores, the parasite was also identified in some aquatic mollusks like mussels [132,133] and oysters [134], which have been used as biomonitors for ecological risk assessment; however, the potential role of mollusks as vectors in transmission of *E. bieneusi* remains elusive due to the lack of molecular typing data. While *E. bieneusi* has been identified in bottlenose dolphins [135], further detailed evidence is needed to determine their infectivity to other hosts.

4. *E. bieneusi* in the environment

E. bieneusi spores can be transported into water bodies and become waterborne pathogens (Fig. 1). The discovery of *E. bieneusi* in surface water can be traced back to the late 1990s [31,136]. A waterborne outbreak of microsporidiosis by *E. bieneusi* was documented in 1999 [14]. Humans and domestic animals are possible sources of *E. bieneusi* contamination in drinking source water, as judged by identification of both host-specific genotypes C (human), EbpB (pig), and PtEb IX (dog) and zoonotic genotypes EbpC, D, EbpA, Peru8, and Peru11 in the rivers impacted by human and livestock activities [36,137]. The negative impacts of *E. bieneusi*-infected wild mammals may extend beyond natural ecosystems to human and livestock communities during storm events; genotypes D and Type IV are commonly found in wildlife within drinking source watershed [37]. Urban storm overflow that contains human-origin genotypes such as D, Type IV, Peru8, and Peru11 can potentially act as a contamination source for drinking source water and recreational water [138]. Beaches, lakes, and rivers used for bathing or yachting can harbor viable *E. bieneusi* spores; bathing in those public waters or direct contact with contaminated water constitutes a potential infection risk [32,139–141]. The contamination of *E. bieneusi* spores in lakes, rivers, and coastal areas may be attributed to urban sewage discharge or agricultural runoff with human and animal wastes [133]. Potentially host-specific (e.g., C and PtEb IX) and zoonotic (e.g., D, EbpC, Type IV, and BEB6) *E. bieneusi* genotypes have been frequently detected in untreated sewages or raw wastewater, which might come from humans and domestic and wild animals; despite sewage treatment, *E. bieneusi* spores persist in the wastewater treatment effluents, thereby posing a threat for the contamination of surface water used for recreation and drinking water abstraction [33,34,38,137,138,142–148].

The contamination of retail fresh food produce such as berries, sprouts, and green-leafed vegetables with potentially viable *E. bieneusi* spores could result from the application of dirty water in the production process, untreated sewage or animal feces to fertilize ready-to-eat crops, and runoff-influenced surface water for crop irrigation (Fig. 1) [39,149,150]. In reality, *E. bieneusi* has caused an outbreak of gastrointestinal illness in a group of apparently immunocompetent persons who visited a hotel in Sweden and consumed cheese sandwiches and salad. Cucumber slices were identified as the most probable vehicle of transmission. The identification of the human-adapted genotype C implicated the potential contamination source to human waste [13].

5. One Health perspectives

Evidence accumulated thus far has shown a common occurrence of *E. bieneusi* in the ecosystem and high genetic diversity of the pathogen among isolates from different hosts and the environment. This has allowed the evolution of diverse genotypes with different host ranges and adaptation to various ecological niches, presenting substantial challenges in the assessment of the sources and public health importance of the small-sized pathogens found in the ecosystem. Molecular ecological studies of *E. bieneusi* have been greatly hampered due to multiple factors, including 1) low discriminatory power or effectiveness in the currently used typing loci, 2) poor understanding of the relationship between genetic variation and phenotypic traits, 3) absence of standardized methods used for differentiating *E. bieneusi* isolates and tracing the routes of infection in epidemiological investigations, and 4) lack of genotype data from humans in many geographical areas of the world. A novel MLST strategy with significantly expanded typing range is urgently needed for *E. bieneusi* to open up new opportunities for solving a central question regarding how genetic variations drive phenotypic traits and their effects on the host and natural environment. In addition, there is a critical need for molecular epidemiological studies conducted on diverse reservoir hosts including humans in the same areas to fully elucidate the cross-species or zoonotic transmissibility of *E. bieneusi*. Some recent studies concerning several other important

protozoan parasites such as *Cryptosporidium parvum*, *Giardia duodenalis*, *Toxoplasma gondii*, and *Leishmania* spp. have already highlighted the value of a One Health approach in helping control the diseases they cause [151–155].

The reservoir hosts of *E. bieneusi* are comprised of a number of epidemiologically connected populations of the same or different species, potentially including vectors. *E. bieneusi* can be maintained by those hosts and transmitted to each other. Domestic and wild animals both represent the well-documented reservoir hosts of *E. bieneusi* and many animal species are infected with both potentially zoonotic and host-specific *E. bieneusi* genotypes. In this context, effort should be directed to search on genetic predictors of *E. bieneusi* adaptation to specific hosts or host switching. *E. bieneusi* infections are frequently asymptomatic in the host species investigated, although clinical disease characterized by diarrhea does occur. These subclinical infections in reservoir hosts may be beneficial to the spread of *E. bieneusi* to other reservoir or susceptible hosts, thus could be a long-term strategy in the coevolution of host-parasite interactions. In light of the absence of safe and effective therapeutic agents and vaccines for *E. bieneusi*, One Health preventive measures should be taken to reduce the close contacts between infected and susceptible hosts and contamination of food and water with *E. bieneusi* spores commonly found in animal feces and human sewage.

Although there is increasing recognition of the major threat of *E. bieneusi* to public health, the ecological complexity of its transmission continues to pose challenges in combating zoonotic infections. Animals are believed to be the major sources of environmental contamination with *E. bieneusi*; spores released from animal stools can be dispersed into water sources, especially via wastewater discharges and farm runoff. Surface water for irrigation contaminated with animal fecal matters represents a latent source for foodborne *E. bieneusi* infection. It may be worthwhile to investigate how surface water and ground water contamination is facilitated by the small size of *E. bieneusi* spores, as there have been no studies on the transport of *E. bieneusi* spore in the environment. The cognition of infection sources and transmission routes, particularly the importance of waterborne or foodborne transmission, could be helpful in the formulation of preventive measures to limit the dissemination of *E. bieneusi* to urban environment. All these call for adopting a One Health approach to the control and prevention of microsporidiosis by *E. bieneusi*, via collaborative and interdisciplinary efforts that span the medical, veterinary, and ecological realms.

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Declaration of Competing Interest

The authors Wei Li and Lihua Xiao declare that no competing interests exist.

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