Environmental DNA in human and veterinary parasitology - Current applications and future prospects for monitoring and control

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ABSTRACT

Parasites are important pathogens with significant global economic, public and animal health impacts. Successful control or elimination of many parasitic diseases, not least neglected tropical parasites, will require scalable, sensitive and cost-effective monitoring tools. Environmental DNA (eDNA) methods, used extensively in ecology for biomonitoring in natural ecosystems, offer promising advantages such reduced costs and labor requirements for species monitoring. Yet, the use of eDNA-based methods in parasitology and disease surveillance, has only recently begun to be explored. With this review, we wish to give an up-to-date overview of current uses and limitations of eDNA in human and veterinary parasitology, and how existing challenges can be overcome to fully utilize the potential of eDNA for monitoring and control of parasitic diseases. We begin by systematically searching published literature to identify studies that apply eDNA methods in parasitology and synthesize the main findings from these studies. We find that eDNA applications in parasitology only account for a small proportion (73/1960) of all eDNA publications up to now, and even fewer (27/73) studies, that apply eDNA methods specifically for parasites of human or veterinary importance. The majority of studies concern snail-borne trematodes and their intermediate host snails, while a few apply eDNA for mosquito vector species detection. A strong geographical bias, with only very few studies undertaken on the African continent, where parasites are of the biggest public health concern, is also noted. Current obstacles hindering further advances of eDNA methods in parasitology include incomplete reference databases, and challenges related to real-time monitoring in remote areas, and in certain LMIC settings. Finally, we point to future opportunities for eDNA-based research in parasitology and highlight recent innovations in eDNA research, which could further develop its application for monitoring and control of parasitic diseases and vectors in the future.

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1. Introduction

Parasites are recognized as important pathogens with major economic, environmental, public and animal health impacts globally (Lustigman et al., 2012). More than three billion people worldwide are estimated to harbor one or more parasites with varying morbidity and mortality (Elsheikha, 2014), with far the biggest burden in low and middle income countries (LMIC) (Hotez, 2018). Parasites are also a major cause of production and disease loss in livestock, causing significant economic loss and with severe impact on animal welfare (Rashid et al., 2019).

Luckily, efforts to rid the world of many of the most important neglected tropical parasitic diseases are becoming increasingly ambitious, proven by a recent paradigm shift that now entails moving from control to elimination (World Health Organization, 2021). Intensified control of many human parasitic diseases in Africa such as schistosomiasis, filariasis, dracunculiasis and cysticercosis has, where successful, led to an expansion of areas with low human prevalence and environmental transmission (Stothard et al., 2017). However, under such scenarios, accurate diagnosis and formal assessment of actual declines or interruption of environmental transmission as 'end game' scenarios arise, is challenged by a lack of reliable tools for environmental transmission surveillance lacking (World Health Organization, 2021).

We are also witnessing how environmental modifications such as climate change, environmental disruption and globalization contribute to a geographical expansion of many parasites and vectors that are sensitive to climatic and environmental changes (Chala and Hamde, 2021). This has increased the rate at which these diseases emerge and spread into new areas including Europe, with little on-going surveillance (Medlock et al., 2012; Rocklöv and Dubrow, 2020). For many of these emerging parasitic diseases, early detection of problematic vector organisms in the environmental phase, will be key for successful early interventions and cost-effective control (Metha et al., 2007).

To further improve control- or elimination efforts of parasites of human and veterinary importance, there is an increasing need for a more precise and formal investigation of the environmental transmission (Stothard et al., 2017). This includes the need to detect or monitor the free-living, infective stages of parasites, as well as the vectors in the environment. In fact, for many human parasitic diseases, such as malaria, dracunculiasis and schistosomiasis the very secret to successful control or elimination, has often proved to lie in targeting both the environmental stages (often through control of the vector) as well as stages within the human host (Ferguson et al., 2010; Sokolow et al., 2018). However, a remaining challenge for a more accurate and timely monitoring of many parasitic diseases, is a lack of scalable, cost-effective and sensitive diagnostic tools for rapid detection of on-going environmental transmission (Bergquist et al., 2017; Sengupta et al., 2019).

Environmental DNA (eDNA) is increasingly being used as a tool for species detection in various environmental substrates, ranging from water, soil to air (Taberlet et al., 2018), potentially offering a strong complement for monitoring of parasitic diseases. In particular, the application of eDNA methods have shown great potential for the study of organisms at low abundance, i.e. rare, elusive or endangered species, as well as in early detection of invasive species and estimation of biodiversity (Deiner et al., 2021; Harper et al., 2019). Since 2008, where a seminal paper on the use of eDNA for aquatic macro-organisms (invasive bullfrog, *Rana catesbeiana*), was published by Ficetola et al. (2008) the number of published papers using or developing eDNA-based methods has increased exponentially (Beng and Corlett, 2020; Veilleux et al., 2021). There seems to be an obvious potential for detection of parasites that encompass some form of environmental transmission stage in their life cycle. Yet, the application of eDNA methods in the field of parasitology and disease surveillance, has only recently begun to be explored (Selbach et al., 2019).

Box 1
Glossary

Environmental DNA (eDNA): Total DNA extracted from an environmental sample (i.e. water, sediment, feces or air) originating from various organisms across taxa (micro and macro) without prior isolation of the target organisms. The eDNA is potentially degraded and present in low concentrations.

Genomic DNA (gDNA): DNA extracted from organism-derived samples (i.e. tissue, blood, feces). It can be from a single organism or a pool of individuals of the same species (e.g. snails, helminths etc).

Invertebrate-derived DNA (iDNA): Vertebrate DNA traces extracted from invertebrates known to feed on vertebrate blood, flesh and/or decaying matter (e.g. ticks, mosquitos, leeches, flies). Considered as part of the eDNA field.

High-throughput sequencing (HTS): Sequencing technique that allows for parallel sequencing of millions of sequences compared to the Sanger sequencing method of processing one sequence at a time. Also known as Next generation sequencing (NGS).

DNA metabarcoding: A method to asses overall biodiversity in a sample, by amplifying DNA in PCR using universal primers that target short DNA fragments that are taxonomically informative. This is followed by next-generation sequencing to generate thousands to millions reads. From this data, multiple species presence is determined. If DNA is extracted from an environmental sample, it is called eDNA metabarcoding (see Fig. 1).

Isothermal DNA amplification: A method where DNA is amplified at a constant temperature, contrary to PCR where the reaction is carried out with a series of alternating temperature steps or cycles.

With this review, we aim to bring to the attention of parasitologists, the potential of eDNA methods as a promising addition to the molecular detection and monitoring toolbox used in parasitology. It is a tool that could prove valuable for improved surveillance and control of environmentally transmitted parasites and vectors onwards. We first investigate current applications of eDNA in human and veterinary parasitology. We do so by systematically searching published literature to identify studies that have applied eDNA methods in parasitology up till now, and synthesize the main findings from these studies. We then point to current limitations and challenges encountered, and how these may be overcome. Finally, we highlight recent innovations in eDNA-based monitoring that with some further development, could be used to fully maximize the potential of eDNA for monitoring and control of parasitic diseases and vectors in the future.

We focus on the application of eDNA for parasites of medical and/or veterinary importance which includes parasitic protozoans, parasitic helminths, and arthropods that directly cause disease (Castro and Olson, 1996) (from here on the term 'parasitology' or 'parasite' refers to parasites of medical or veterinary importance including zoonoses, if nothing else is stated). Vectors, a third party transmitting or carrying the parasites, such as mosquitoes, ticks, flies and snails, are also included in this review since many parasites with public and animal health importance are vector borne.

2. What is eDNA really? Getting to grips with the terminology

The definition of eDNA used in the field of ecology and conservation considers both organismal DNA, i.e. from whole individuals, and extra-organismal, i.e. shed tissue, free DNA, present in an environmental sample such as water, soil, sediment, feces or air (Pawlowski et al., 2020; Rodriguez-Ezpeleta et al., 2021). Contrary to DNA extracted directly from fresh tissue (genomic DNA; Box 1), eDNA is characterized by a complex mixture of nuclear, mitochondrial and chloroplast DNA originating from different organisms which have been shed from feces, urine, skin, hair, gamets and cells into the environment (Taberlet et al., 2012). Thus, eDNA samples constitute intracellular DNA, but also degraded extracellular DNA (often short fragments) because of cell rupture and natural degradation in the environment (Taberlet et al., 2012). Importantly, eDNA is thus defined by the medium where it is found and not by its taxonomic composition or specific structural state (intra- or extracellular) (Pawlowski et al., 2020). A recent development within the field of eDNA, is detection of vertebrate DNA traces in invertebrates that feed on vertebrate blood, flesh, dead or decaying organic matter. This is termed invertebrate-derived DNA (iDNA; Box 1) (Calvignac-Spencer et al., 2013). Due to their feeding behavior, these invertebrates such as mosquitoes, leeches and flies, gather vertebrate DNA, and the main purpose of iDNA studies have been to assess vertebrate diversity in general or monitor rare vertebrates, which appear in low abundance or have shy behavior (Lynggaard et al., 2019; Schnell et al., 2015).

Since the first introduction of the term 'environmental DNA' in the late 1980's applied to the detection of microorganism DNA in environmental samples (Ogram et al., 1987; Somerville et al., 1989) until its current use, an important milestone was the detection of eDNA from a macro-organism, the bullfrog *Rana catesbeiana*, in freshwater samples (Ficetola et al., 2008). Then followed the development of new sequencing technologies, such as High-throughput sequencing (HTS; Box 1) which allowed parallel processing of many samples (Bennett, 2004; Margulies et al., 2005) and opened new avenues for generating full biodiversity profiles (many taxa) of ecosystems based on environmental samples, i.e. eDNA metabarcoding (Deiner et al., 2017; Box 1). Determining presence or absence of a single taxon in eDNA samples requires species-specific primer-probe assays application via PCR or quantitative PCR (qPCR) often combined with Sanger sequencing (i.e. Knudsen et al., 2019; Takahara et al., 2020) (Fig. 1). In contrast, for eDNA metabarcoding universal primer sets targeting a conserved homologous region of a gene shared by several species or taxa groups is used (i.e. Alexander
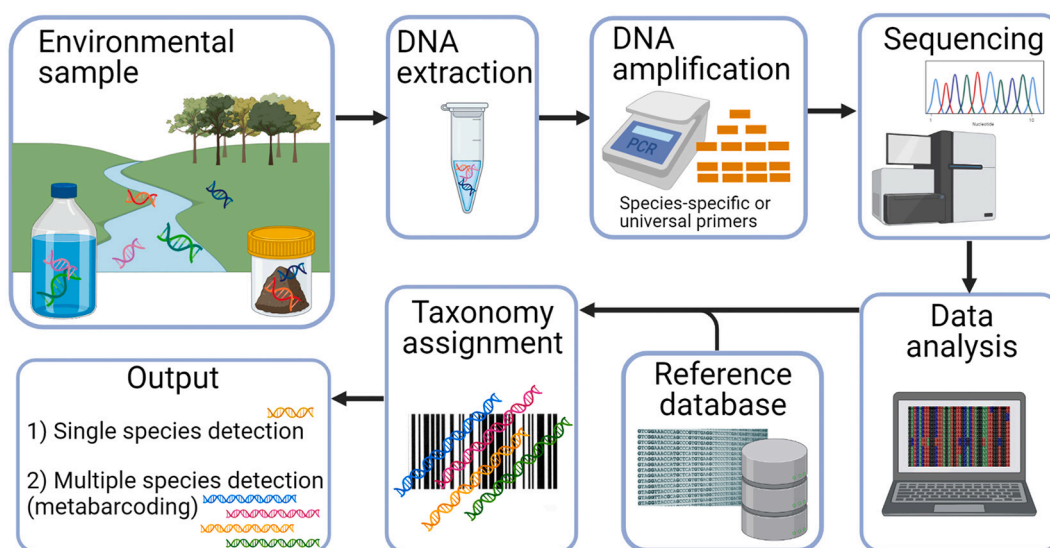

Fig. 1. Overview of the eDNA workflow from field sampling to species detection.

et al., 2020; Thomsen and Sigsgaard, 2019). The gene region is then amplified via PCR and sequenced on a HTS platform (Box 1) from where millions of DNA sequences are generated, then bioinformatically processed and compared to reference databases to assign taxonomic identifications (Fig. 1). For either approach, the choice of assay depends on reference sequence availability and target locus availability and suitability in the taxon/taxa of interest (Beng and Corlett., 2020). When developing eDNA assays important steps for primer validation should be considered, including: in silico (desktop-based) validation of primers against reference sequences of closely related species; in vitro (lab-based) validation against tissue-derived or synthetic DNA of both target and related species; and field-based in situ validation as the final step (a controlled environment harboring the target organism can be included before going to the field). More details for each step in designing eDNA studies can be found for instance in Goldberg et al. (2016) and Bruce et al. (2021).

DNA-based methods for parasite detection in environmental samples is common and undergo a fast development in parasitology, i. e. for detection of *Cryptosporidium/Giardia* spp. in water samples (Rochelle et al., 1997), *Toxoplasma gondii* in water and soil samples (Afonso et al., 2008; Aramini et al., 1999; Lass et al., 2009) or soil-transmitted helminths in fecal samples (Pecson et al., 2006; Verweij et al., 2001). However, the term 'environmental DNA' has typically not been used in this field. This demonstrates a seemingly substantial overlap between the commonly applied molecular methods in parasitology and the 'eDNA approach' as it is defined by ecologists (Bass et al., 2015). It warrants some clarification of terminology with specifications of when DNA-based detection is considered "eDNA", and when not, as this often leads to confusion in the cross-field between "ecology eDNA" and "parasitology eDNA" studies. Fig. 2 illustrates this overlap and transition between more classical molecular methods applied in parasitology and the eDNA methods with indication of which purpose the methods are most suitable for.

In medical and veterinary parasitology, the purpose of a study such as individual diagnosis, treatment efficacy evaluation or research decides the most suitable method for parasite detection inside a host or outside in the environment. It likewise has an influence on the appropriate sample types needed, regardless of being strictly defined as eDNA or not. However, the eDNA approach is by definition different from conventional molecular methods in parasitology, and has certain features and advantages, not currently covered by other methods in parasitology. These are outlined in Fig. 3. Importantly, with eDNA methods, there is no need for the typically time-consuming and laborious visual taxonomic identification of the target organism itself (Taberlet et al., 2012). This is an obvious advantage in parasitology where several species and stages of parasites and vectors often are small and elusive and thus difficult to identify for the untrained. The need for specialized knowledge on taxonomy and specimen morphology, otherwise mandatory, is thus of less importance during sampling using eDNA, and allows for a more extensive sampling to be undertaken by others than trained experts. Finally, the possibility of detecting multiple species from the same environmental sample improve temporal and spatial monitoring due to reduced time in the field and may render eDNA methods alternatives or supplements to the toolbox currently applied in parasitology.
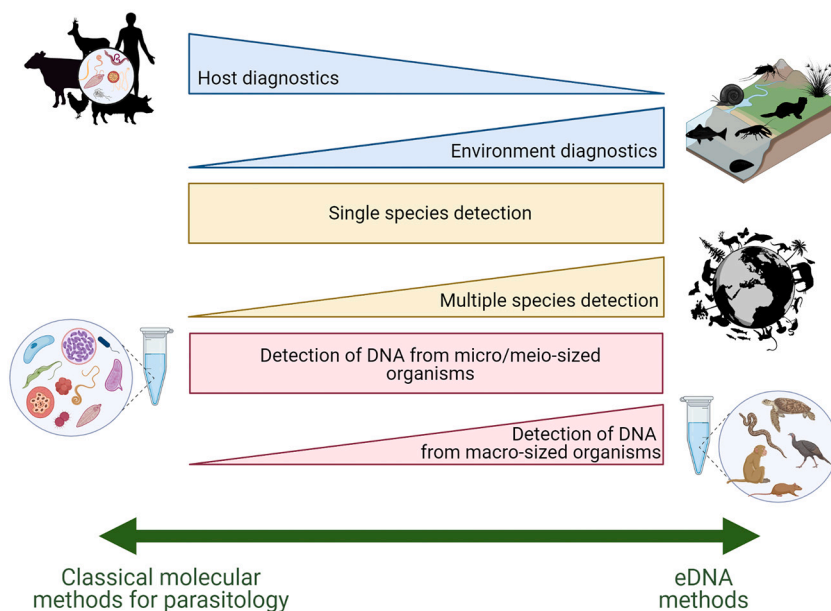

Fig. 2. The continuum and overlap between classical molecular methods used in parasitology (left side) and eDNA methods (right side) with indication of main purpose or suitability. Top two bars (blue): Diagnostics of parasites in the host (e.g. human, livestock) vs. in the environment (i.e. free-living parasite stages, early detection of invasive/cryptic species, biodiversity assessment). Middle two bars (yellow): The number of species detected in the sample, going from a single species to multiple species, where total biodiversity is detected simultaneously. Bottom two bars (red): The size of the target organism ranging from micro-sized organisms (i.e. parasites and bacteria, where the whole organism is potentially present during sampling, i.e. water, feces) to macro-sized organisms (i.e. large vertebrate animals where only the DNA traces left behind is detected).
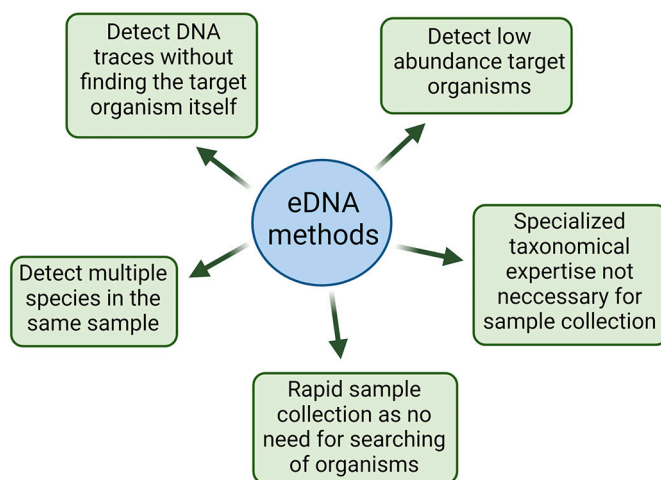
Fig. 3. The advantages of eDNA methods which could be very useful for application in parasitology.

3. Current applications of eDNA in human and veterinary parasitology and disease surveillance

3.1. Literature review methodology

To get an overview of current applications of eDNA in parasitology, a search of all available published literature in the period 1 January 2008 to 31 December 2021 was conducted in the Web of Science and PubMed databases. It was beyond the scope of this review to classify all the parasitology studies that apply an eDNA workflow, but not the terminology 'environmental DNA' or 'eDNA' as this is a review in itself. We therefore caution that this review is non-exhaustive in the context of molecular parasitological diagnostics for e.g. feces. It does however, provide an update of the current trends, emerging research and future directions within the application of eDNA for monitoring and control for parasites (and vectors) of human and veterinary importance. Two searches were conducted. The first was to identify the total number of eDNA studies in published literature and the second to specify the proportion and number of studies where eDNA has been used in connection with the study of parasites, pathogens or disease vectors. The first search used the term ('eDNA' OR 'environmental') AND 'DNA'). The second search used the search terms ("environmental DNA" OR "eDNA") AND (pathogen* OR disease* OR parasit* OR zoonotic* OR vector*). The first search (for all eDNA studies) gave 1960 results, while the
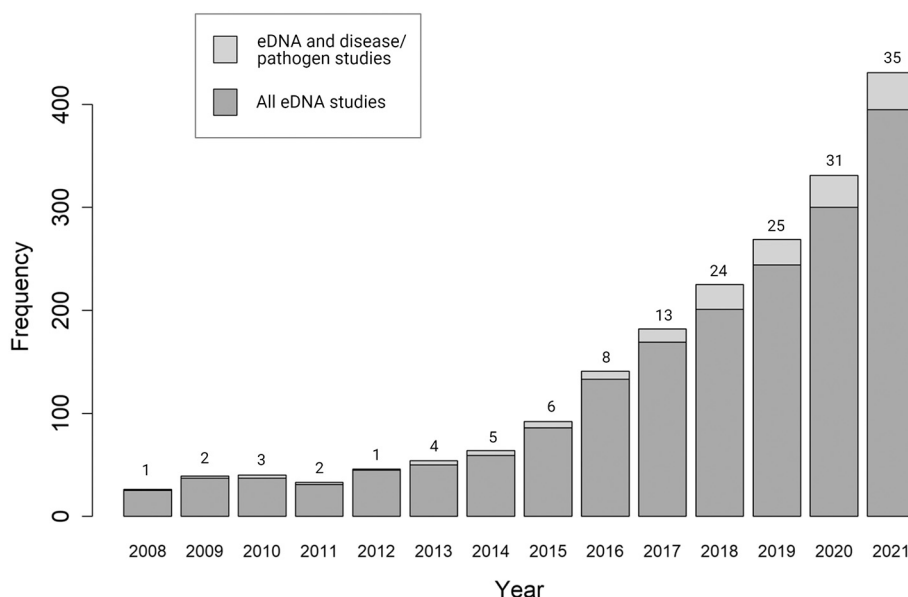

Fig. 4. The number of publications by year referring to eDNA and any human and/or animal pathogen or disease (light grey) out of all eDNA studies (dark grey) for the period 1 January 2008 to 31 December 2021. The number above each bar refers to the number of publications on eDNA and disease/pathogen studies each year.

second search yielded a total of 887 publications (after duplicates were removed). These papers were then screened by reading through the titles and abstracts (independently by two of the authors) and narrowing down by first excluding full-text articles that did not meet the inclusion criteria (i.e. not about eDNA and parasites, pathogens or diseases of any kind). The remaining papers from the second search, were then screened in more detail (full text) and categorized into papers specifically concerned with eDNA, parasites, and vectors, and finally papers that deal specifically with eDNA and parasites or vectors of human and/or veterinary importance. A detailed schematic overview of the criteria and number of papers excluded/included can be seen in the PRISMA flow diagram of the eligible study selection process in Fig. S1.

3.2. Results from literature review

After the first screening, excluding non-relevant studies, we identified 160 papers published in the period 2008–2021 that concerned the use of eDNA in studies of any human and/or animal pathogen or disease. Fig. 4 shows the trend of these papers by year of publication. The number of all published papers on eDNA is included as a background to illustrate the exponential development in eDNA studies in general. Of these 160 papers, 73 papers dealt specifically with parasites and/or vectors/intermediate hosts of a parasite and eDNA applications (3.7% out of all published eDNA studies). Among these, the majority of studies were related to studies on eDNA and human parasites or (non-human) fish parasites (mainly in aquaculture). The studies broken down by final host type can be seen in Fig. 5a. When the 73 papers were distributed according to geographical region (defined as where the study was done), it was evident that most of the studies are from Europe followed by USA (Fig. 5b), while very few are from for example Africa and Asia, regions where parasites typically pose the greatest human health challenge.

As the main focus of this review concerns the use of eDNA in human and veterinary parasitology, our final screening aimed to identify studies that specifically use eDNA methods in connection with human and zoonotic parasites as well as relevant vectors of parasites (see Fig. S1). This resulted in 36 studies, of which 27 were original research papers (Table 1) while nine papers were reviews or commentaries, that did not necessarily apply eDNA as such but merely referred to other studies doing so. The current applications of eDNA for detection of human and veterinary parasites (Table 1), has mainly focused on trematodes of the genera *Schistosoma, Trichobilharzia, Opistorchis* and *Fasciola* (Alzaylaee et al., 2020a; Jones et al., 2018; Rudko et al., 2019; Sato et al., 2018; Sengupta et al., 2019). These are all snail borne parasites with free-living transmission stages in the environment, thereby being detectable with eDNA-based methods. For vectors, eDNA has been applied both for the snail hosts of the snail borne trematodes (Jones et al., 2018; Rathinasamy et al., 2018) and for mosquitoes as potential vectors of protozoan or filarial parasites (Krol et al., 2019; Schneider et al., 2016). The environmental sample types collected across the 27 studies are mainly freshwater, whereas for snail hosts soil or sediment has also been used. One recent study utilized mosquito blood meals to detect vertebrate reservoir species and is the only application of iDNA found in this literature search (Hopken et al., 2021). The two eDNA analysis approaches applied in the studies in Table 1 are either single-species detection or assessment of total number of taxa (metabarcoding) (Box 1; Fig. 1), which are also the main approaches applied in ecology and conservation studies. The eDNA studies presented in Table 1 have used both newly designed and validated primers targeting the taxon/taxa of interest and primers designed for other purposes, but validated for eDNA detection. In Table 2, an overview is provided of target genes and primers applied specifically for parasite or vector species detection in eDNA studies published in 2008–2021.

4. Main challenges and possible solutions

Even though eDNA-based methods offer the potential for improved detection of some parasitic diseases, it is important to be aware of limitations of the methods when compared to already established, more traditional methods used in parasitology for species

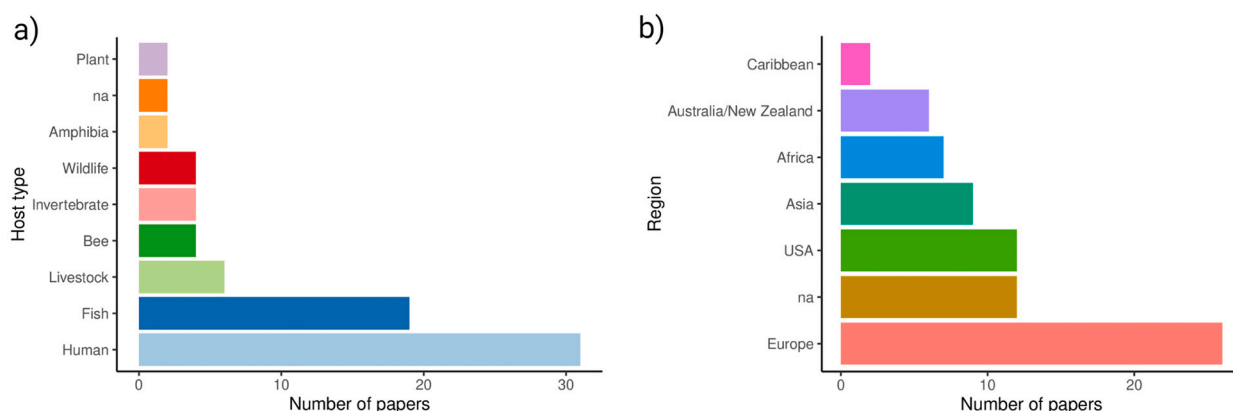**Fig. 5.** The distribution of the 73 studies on eDNA and parasites according to a) the main host organism, and b) geographical region. For host type: 'Invertebrates' include other species than vector/intermediate host of parasites, which are included in Human. The papers on zoonotic parasites are part of Livestock, na = non-specific host type. For region: na = not applicable (review or only laboratory based).

Table 1
Overview of the studies using eDNA in human and/or veterinary parasitology (2008–2021).

Parasite or vector/snail-host	Target taxon/taxa	Environmental sample type	Detection approach	Reference
Parasite	*Schistosoma japonicum*	Freshwater (spiked)	qPCR	(Hung and Remais, 2008)
Parasite	*Trichobilharzia* sp.	Freshwater	PCR	(Schets et al., 2010)
Parasite	*Trichobilharzia* sp.	Freshwater	qPCR	(Jothikumar et al., 2015)
Vector	*Aedes* sp. / Culicidae	Freshwater	qPCR / metabarcoding	(Schneider et al., 2016)
Vector	Arthropod vectors	Dust	Metabarcoding	(Madden et al., 2016)
Parasite	*Opistorchis viverrini*	Freshwater	qPCR	(Hashizume et al., 2017)
Parasite	*Schistosoma mansoni*	Freshwater	qPCR	(Sato et al., 2018)
Parasite	*Trichobilharzia* sp.	Freshwater	qPCR	(Rudko et al., 2018)
Parasite + Snail-host	*Fasciola hepatica* + *Galba truncatula*	Freshwater	PCR	(Jones et al., 2018)
Parasite + Snail-host	*Fasciola* + *Austropeplea tomentosa*	Freshwater	Multiplex qPCR	(Rathinasamy et al., 2018)
Vector	*Anopheles gambiae s.l.*	Freshwater	qPCR	(Odero et al., 2018)
Parasite	*Schistosoma mansoni*	Freshwater	qPCR	(Sengupta et al., 2019)
Parasite	*Trichobilharzia* sp. – 4 species	Freshwater	qPCR	(Rudko et al., 2019)
Snail-host	*Oncomelania hupensis quadrasi*	Soil	qPCR	(Calata et al., 2019)
Vector	Culicidae	Freshwater	qPCR / Metabarcoding	(Krol et al., 2019)
Vector	Culicidae	Freshwater and sediment	Metabarcoding	(Boerlijst et al., 2019)
Parasite + Snail-host	*Schistosoma japonicum* *Oncomelania hupensis quadrasi*	Freshwater	qPCR	(Fornillos et al., 2019)
Snail-host	*Bulinus truncates*	Freshwater	qPCR / ddPCR	(Mulero et al., 2019)
Parasite	*Schistosoma mansoni* *Schsitosma haematobium* *Schistosoma japonicum*	Freshwater	qPCR	(Alzaylaee et al., 2020a)
Parasite	*Schistosoma mansoni* *Schsitosma haematobium*	Freshwater	qPCR	(Alzaylaee et al., 2020b)
Parasite	*Schistoma haematobium*	Freshwater	qPCR	(Eyre et al., 2020)
Parasite	*Trichobilharzia* sp.	Freshwater	qPCR	(Rudko et al., 2020)
Snail-host	*Galba truncatula*	Freshwater	LAMP	(Davis et al., 2020)
Parasite + Snail-host	*Fascila* + *Austropeplea tomentosa*	Freshwater	Multiplex qPCR	(Rathinasamy et al., 2021)
Snail host	*Galba truncatula*	Freshwater	PCR	(Jones et al., 2021)
Snail host	Gastopoda	Freshwater	Metabarcoding	(Mulero et al., 2021)
Reservoir host	Vertebrata	Mosquitos (iDNA)	Metabarcding	(Hopken et al., 2021)

detection, disease monitoring and surveillance. A number of potential pitfalls are related to the collection and data interpretation of environmental samples for parasitic disease surveys. When applying eDNA methods the general technical challenges covers contamination, false positives, false negatives, and eDNA degradation, all reviewed elsewhere (Beng and Corlett, 2020; Goldberg et al., 2016; Harper et al., 2019). These limitations may obviously also challenge eDNA techniques applied in parasitology and are thus summarized in Table 3. Especially, the inability of eDNA to distinguish between different life-stages of the same species pose a challenge in parasitology. For example, differentiating eDNA signals from free-living miracidia and cercariae of *S. mansoni* is important in order to assess whether there is a risk of contamination (miracidia are only infective for the snail hosts) or risk of exposure (cercariae are infective for the final host, i.e. humans) (Sengupta et al., 2019). Though not in the near future, targeting mRNA genes in environmental samples (e-mRNA) could perhaps differentiate parasite life-stages and have gained more traction lately with detection of tissue-specific eRNA in zebrafish tank water (Tsuri et al., 2021). Within reach is detection of environmental RNA (eRNA) which could improve environmental monitoring since i) RNA can differentiate between living (metabolically active) and dead cells (Cristescu, 2019), and ii) higher instability of RNA as compared to DNA allows for faster eRNA degradation resulting in more accurate and real-time (spatiotemporal) species detection (Laroche et al., 2017).

The possibility of correlating eDNA concentrations with the abundance and/or biomass of aquatic organisms is still being investigated across taxa (Doi et al., 2016; Goldberg et al., 2013; Pilliod et al., 2013). These correlations are still coupled with a high degree of uncertainty due to our limited overall understanding of eDNA dispersal and degradation (Rourke et al., 2022; Stewart, 2019). Several of the studies on application of eDNA methods parasitology (see Table 1) quantify the concentration of the detected eDNA and correlate the amount of eDNA with parasite abundance or density (Rathinasamy et al., 2021; Rudko et al., 2018). However, it is currently not possible to translate for example number of cercariae/L of water into a measure of risk of infection. Therefore for full utilization of abundance estimates based on eDNA concentrations, more research is needed on the links between parasite eDNA presence or absence, parasite abundance, environmental transmission of the parasite (infection risk), and the number of infected people or animal final hosts.

A fundamental challenge in relation to the application of eDNA methods for many species of parasites and vectors, is that the genes sequenced and available in public reference databases, i.e. NCBI and BOLD, are highly patchy (Krol et al., 2019; Mulero et al., 2021). Often only a single locus (e.g. the typical barcode region of COI (Hebert et al., 2003)) or few loci are sequenced, and if the molecular taxonomic resolution of the targeted gene(s) is too low to separate closely related species the development of species-specific primers suitable for eDNA use is limited. This is seen in *Biomphalaria* snail species, the vector snail of *S. mansoni* trematodes, where partially sequences of COI, 16S, and ITS1genes are not able to differentiate the species (Joergensen et al., 2007). Currently, eDNA primers for *Biomphalaria* species detection has not been successfully developed, whereas eDNA assays for the other vector snail species, *Bulinus truncatus* (Mulero et al., 2019) and *Oncomelania hupensis quadrasi* (Calata et al., 2019; Fornillos et al., 2019), as well as the three main

Table 2

Overview of primers applied for parasite or vector detection in eDNA studies published in 2008–2021.

Target taxa	Target gene	Primer name (F/R/P)[a]	Primer sequence (5′-3′)	Amplicon size (bp)	Validated for	Reference[b]
Trematodes						
Schistosoma mansoni	COI	Sma-COI-F (F)	CAGGGGTTTCAAGTCTAATTGGAT	162	Field collected water	(Sato et al., 2018)
		Sma- COI-R (R)	CAAATAATAACATCGTTATTCCTCTGG			
		Sma-COI-P (P)	FAM-TTCAAATGTTCGATAATA-NFQ-MGB			
	COI	Schiman_COIF (F)	ATTTACGGTTGGTGGTGTCA	86	Field collected water	(Sengupta et al., 2019)
		Schiman_COIR (R)	GAGCAACAACAA ACCAAGTATCA			
		Schiman_COIprobe (P)	FAM-GGGGTGGCTTTATCTGCATCTGC-BHQ-1			
	16S	SM-16SrRNA-F (F)	CTGCTCAGTGAAGAAGTTTGTTT	104	Field collected water	(Alzaylaee et al., 2020a, 2020b)
		SM-16SrRNA-R (R)	CCTCATTGAACCATTCACAAGTC			
		SM-16SrRNA-P (P)	FAM-AGCCGCGATTATTTATCGTGCTAAGGT-BHQ-1			
Schistoma haematobium	16S	SH-16SrRNA-F (F)	AATGAACATGAATGGCCGCA	143	Field collected water	(Alzaylaee et al., 2020a, 2020b)
		SH-16SrRNA-R (R)	ATGGGTTCCTCACCACTTAAACT			
		SH-16SrRNA-P (P)	FAM-TGGAGACTTGTGAATGGTCGAACG-BHQ-1			
Schistosoma japonicum	Putative deoxyribodipyrimidine photo-lyase	PL-F (F)	GCCTTCTTGTTTGCTCAACGT	85	Spiked water	(Hung and Remais, 2008)
		PL-R (R)	CCGCTTGATATTTTGGAACGA			
		PL-PR (P)	FAM-TAGCGTTAAAATTTAAAGTCCCTCTCCATGTTTGTTTCT–TAMARA			
	COI	Sj_COI_F (F)	TTTGATAACTAATCACGGTATAGCAA	119	Field collected water	(Fornillos et al., 2019)
		Sj_COI_R (R)	CGAGGCAAAGCTAAATCACTC			
		S. japonicum (P)	FAM-TTTTGGTAAATATCTTCTTCCG-MGB-NFQ			
Schistosoma genus	ITS-2	Ssp48F (F)	GGTCTAGATGACTTGATYGAGATGCT	77	Field collected water	(Eyre et al., 2020; Obeng et al., 2008)
		Ssp124R (R)	TCCCGAGCGYGTATAATGTCATTA			
		Ssp78T (P)	FAM-TGGGTTGTGCTCGAGTCGTGGC-BHQ-3			
Opistorchis viverrini	COI	OV-COI-F (F)	GCTGGATTTGGGCACCG	103	Field collected water	(Hashizume et al., 2017)
		OV-COI-R (R)	AGTACCCGCAAGCATATACAACC			
		OV-COI-P (P)	FAM- TAGCTCGGTTACTATGATTAT-NFQ-MGB			
Trichobilharzia genus	18S	JVSF (F)	AGCCTTTCAGCCGTATCTGT	154	Field collected water	(Jothikumar et al., 2015)
		JVSR (R)	AGGCCTGCCTTGAGCACT			
		JVSP (P)	FAM-TCGGGAGCGGACGGCATCTTTA-BHQ-1			
Trichobilharzia stagnicolae	COI	SRTS FWD (F)	ATTATCTAATTACTAATCATGGGATTGCA	65	Field collected water	(Rudko et al., 2019)
		SRTS REV (R)	ATGCCAAATCATCTAAACCCAAC			
		SRTS PRB (P)	FAM-ACCAAACCC/ZEN/ACCAATCAATACAGGCA-IABkFQ			
Trichobilharzia szidati	COI	SRTSZ FWD (F)	GTTGTTGGGTTCTGTTAAATTTATAAC	42	Field collected water	(Rudko et al., 2019)
		SRSZ REV (R)	AGACGTAAACAAATACGCCCA			
		SRSZ PRB (P)	FAM-TCTTAGTTC/ZEN/TCGGGTTTCGGTTGTTGTT-IABkFQ			

Table 2 (*continued*)

Target taxa	Target gene	Primer name (F/R/P)[a]	Primer sequence (5′-3′)	Amplicon size (bp)	Validated for	Reference[b]
Trichobilharzia physellae	COI	SRTP FWD (F)	TGGTTTGGTWTGTGCTATGGG	51	Field collected water	(Rudko et al., 2019)
		SRTP REV (R)	AKTCTTAACATCCAATCCY			
		SRTP PRB (P)	FAM-TGAGC + TCA + TACTACACTACC + TAAAC-IABkFQ			
Anserobilharzia brantae	COI	SRAB FWD (F)	GATTCCTTCAGAGATTTATAAATATTTA	98	Field collected water	(Rudko et al., 2019)[c]
		SRAB REV (R)	ACGAGGTAACGCCAAATC			
		SRAB PRB (P)	FAM-TACCAAACC/ZEN/CRCCAATRAACACRGGCA-IABkFQ			
Fasciola hepatica	ITS2	Primer F (F)	CCC CTA GTC GGC ACA CTT A	169	Field collected water	(Jones et al., 2018)
		Primer R (R)	TAT GAA ANT AGC ATC AGA CAC ATG A			
	ITS2	qFhITS2 FP (F)	GGTTGGTACTCAGTTGTCA	108	Field collected water	(Rathinasamy et al., 2021, 2018)
		qFhITS2 RP (R)	CAAAGTGACAGTGACGGAA			
		qFhITS2 P (P)	FAM-CCTAGTCGGCACACTTATGATTTCTG-BHQ-1			
Calicophoron daubneyi	ITS2	Primer F (F)	GGGTGTGGCGGTAGAGTC	100	Field collected water	(Jones et al., 2018)
		Primer R (R)	CGGACRGCAATAGCATCTCAA			
Snail vectors						
Bulinus truncatus	COI	Btco1F (F)	TYGAAGGAGGGGTTGGAACA	179	Field collected water	(Mulero et al., 2019)
		Btco1R (R)	RKTRATTCCTGGTGCYCGT			
	COI	Btco2F (F)	ATTTTGACTTTTACCACCAT	165	Field collected water	(Mulero et al., 2019)[d]
		Btco2R (R)	GATATCCCAGCTAAATGAAG			
		Btco2P (P)	TCGAAGGAGGGGTTGGAACAGGFAM			
Oncomelania hupensis quadrasi	COI	OhqCOX1_22-41aF (F)	GCATGTGAGCGGGGCTAGTA	187	Field collected water and soil	(Calata et al., 2019; Fornillos et al., 2019)
		OhqCOX1_189-209aR (R)	AAGCGGAACCAATCAGTTGCC			
		OhqCOX1_67-86P (P)	FAM-GTGCAGAGTTAGGTCAGTCCTMGB-NFQ			
Galba truncatula	ITS2	Primer F (F)	GTGAGCTCTCACGCTGCTC	288	Field collected water	(Jones et al., 2018)
		Primer R (R)	TAGAGCCCCTTGTTCTCCA			
	ITS2	F3	CTCGGCGATGGTTGGATA	18	Field collected water	(Davis et al., 2020)[e]
		B3	ATCTCGTCCGATCTGAGG	18		
		FIP	CCGAGAACGCCACGATAATTGTCCGTTCATCTCGTAAC	28		
		BIP	AGTCCATGGCATCGCAGCACCACGTAGCGTCTTAGA	36		
		LoopF	CTGCCTGGCGGTAGAGAA	18		
		LoopB	GTGGGTGGAGAACAAGGG	18		
Austropeplea tomentosa	ITS2	qAtITS2 FP (F)	GCCAAATTTTCCTCCTCGT	118	Field collected water	(Rathinasamy et al., 2021, 2018)
		qAtITS2 RP (R)	AAGCGAGCGTCAGCGTAA			

Table 2 (*continued*)

Target taxa	Target gene	Primer name (F/R/P)[a]	Primer sequence (5′-3′)	Amplicon size (bp)	Validated for	Reference[b]
Gastropoda	16S	qAtITS2 P (P) Gast01F (F) Gast01R (R)	HEX-CTAACGGGCCCGCTCGTAACA-BHQ-1 CCGGTCTGAACTCAGATCA TTTGTGACCTCGATGTTGGA	60–70	Field collected water	(Mulero et al., 2021; Taberlet et al., 2018)
Mosquito vectors						
Aedes albopictus	ITS1	ITS1_F440 (F) ITS1_R510 (R) AlboITS1P (P)	GTCAGCAGGGCCGAACC GACGACCCGCCACTTAGCT FAM-CAGGGCACATACGTCCGCTTTGGTT-TAMRA	35	Field collected water	(Hill et al., 2008; Schneider et al., 2016)
Aedes japonicus japonicus	COI	COI_F041_japo (F) COI_R141_japo (R) JapoCOIP (P)	GCTCCAGATATAGCTTTCCCTCG GGATAAACAGTTCATCCAGTTCCAG FAM-ACCTTACTACTTTCAAGTAGAATG-MGB	77	Field collected water	(Schneider et al., 2016; van de Vossenberg et al., 2015)
Aedes koreicus	COI	Akore-f (F) Akore-r (R) Akore-probe (P)	CCCAGATATAGCCTTCCCCCG GGATAAACAGTTCAT CCTGTCCCAG FAM-CTCCCTCATTAACTCTACTACTTTCAAGAAGTA TAGTAG-BHQ1	77	Field collected water	(Schneider et al., 2016)
Anopheles gambiae s. l. *Anopheles gambiae.* *Anopheles arabiensis*	28S (IGS)	Primer F (F) Primer R (R) Probe (P) Probe (P)	GTGAAGCTTGGTGCGTGCT GCACGCCGACAAGCTCA VIC-CGGTATGGAGCGGGACACGTA 6FAM-TAGGATGGAGAAGGACACTTA	131	Lab-based tank water	(Odero et al., 2018; Walker et al., 2007)
Culicidae	16S	Culicidae-f (F) Culicidae-r (R)	ACGCTGTTATCCCTAAGGTAACTTA GACGAGAAGACCCTATAGATCTTTAT	146	Field collected water	(Schneider et al., 2016)
Culicidae	COI	eCul-F (F) eCul-R (R)	GGRKCHGGDACWGGDTGAAC GATCAWACAAATAAAGGTAWTCGATC	154	Field collected water, sediment	(Boerlijst et al., 2019; Krol et al., 2019)
Reservoir hosts (mosquitoes)						
Vertebrata	12S	12SV5F (F) 12SV5R (R)	TAGAACAGGCTCCTCTAG TTAGATACCCCACTATGC	110	Mosquito blood meal	(Hopken et al., 2021; Riaz et al., 2011)

[a] The primer is indicated as forward (F), reverse (R) or probe (P).

[b] When the eDNA study have applied a primer-set developed by others, the original reference of that primer-set is also provided.

[c] The plus signs (+) indicate a locked nucleic acid at the preceding nucleotide position.

[d] The primers are developed for digital droplet PCR (ddPCR).

[e] The primers are developed for LAMP assay.

Table 3
Limitations and challenges of eDNA methods.

Limitations/challenges	Description
False positives: (type I error; eDNA detected where target species is not present)	The target eDNA signal could stem from other sources, i.e. dead target organisms, sewage/wastewater, contaminated equipment or could have been transported to the sampling site from other places with e.g. flowing water (horizontal transportation).
False negatives (type II error; eDNA not detected where target species is present)	Differences in target organism's biology (ecology and physiology) can result in uneven spatial and temporal distribution of eDNA in the environment, and the collected environmental sample might just not have 'captured' the target eDNA. Alternatively, the abundance of the target organism is below the lower detection threshold (sensitivity limit) of the eDNA assay.
The inability of eDNA to distinguish between different life-stages.	Currently, eDNA methods are not able to differentiate the eDNA traces from different life stages of the same species.
The inability of eDNA to distinguish between live and dead target organisms.	After being shed into the environment, the persistence of eDNA will be influenced by abiotic (i.e. temperature, pH) and biotic (i.e. microbial activity) factors affecting the rate at which eDNA is degraded. Thus, eDNA can be traceable even after the live target organism is no longer present or dead.
Uncertainty in target organism abundance estimates based on eDNA concentrations.	Apart from detecting a target organism by presence/absence using eDNA methods, quantification of eDNA concentrations (e.g. DNA copies/L of water) is possible. Currently, studies are attempting to translate eDNA concentrations to organism densities, biomass or abundances, e.g. fish biomass in marine environments.

Schistosoma species (Alzaylaee et al., 2020b; Fornillos et al., 2019; Sengupta et al., 2019) have been developed recently. An overview of the published and validated eDNA primers used for parasites and vectors of medical and veterinary importance is provided in Table 2. To immediately overcome the incompleteness of databases, construction of customized DNA reference-databases for single gene-regions using generic primers (e.g. barcode primers) based on tissue-samples can be a solution. Krol et al. (2019) COI-barcoded and Sanger sequenced mosquito specimen of 38 taxa before onset of eDNA primer development. An alternative approach could be sequencing of parasite and vector full genomes so all gene regions would be known, however, this work has just recently begun for a few species i.e. *Schistosoma mansoni*, *S. haematobium* and *Ascaris suum* (Berriman et al., 2009; Jex et al., 2011; Young et al., 2012). Furthermore, the lack of database sequence coverage also hampers the full exploitation of eDNA metabarcoding approaches in parasitology as it can be difficult to assign many of the sequences generated from HTS (see Box 1) to genus or species level (Mulero et al., 2021). Recently, eDNA reference databases are being built as seen in the Danish project 'DNAmark', where DNA from animals and plants occurring in Denmark, including *Fasciola hepatica* liver flukes and their intermediate host snail *Galba truncatula*, is sequenced and made publicly available enabling future eDNA-based environmental monitoring (Margaryan et al., 2021).

Lastly, application of eDNA methods for NTD monitoring is challenged by the scarcity of molecular laboratories and capacity in most countries where the NTDs are endemic. This is an obstacle for eDNA sample processing and minimize the use of the methods in low- or middle-income countries. This can to some extend be alleviated by centralizing the laboratory-part of the eDNA analysis workflow (see Fig. 1) to hospitals, universities or similar institutions with molecular laboratory facilities. The smaller field stations or local health clinics could collect the environmental samples and preserve with e.g. RNAlater which conserves and protects the DNA at room temperature (Spens et al., 2017). The samples can then be transported to central laboratories for final analysis. With respect to costs, DNA analyses are not as cheap as most traditional parasitological methods with regards to reagents and equipment needed. However, in general the cost of molecular methods have decreased over the years due to the overall developments and manufacturers competition. On the other hand, application of the traditional detection methods require personnel with knowledge on taxonomy of e. g. snails and the diseases they transmit. This capacity is unfortunately declining globally (Adema et al., 2012) which underlines the need for reliable monitoring tools. Finally, there is a need for some guiding principles on how eDNA-based surveillance data on medically and veterinary important pathogens can be used in a policy setting by WHO or World Organization for Animal Health.

5. Emerging themes and future prospects for eDNA applications in parasitology

The characteristic of eDNA (non-invasive, scalable, sensitive and in some cases also cost-effective compared to traditional monitoring tools, as outlined in Fig. 3), makes it very suitable for applications in parasitic disease surveillance and mapping today. Indeed, we found that eDNA is already aiding parasitologists in the detection of many water-borne trematodes with environmental stages, intermediate host snails and mosquito vectors (Table 1). Yet, we find that the proportion of studies in human and veterinary parasitology that has so far used eDNA is still relatively small (Table 1, Fig. 4). Below we outline scenarios where eDNA-based methods may already now (with current technologies) be expanded to utilize its full potential in parasitology, but also list research topics (new areas in eDNA research), that given further development, could help overcome some of the current challenges highlighted in section 4.

One appealing advantage of eDNA is the potential for scaling up sampling for monitoring and surveillance. In a warmer future where parasites and vectors are expected to emerge into new territories at faster rates (Stensgaard et al., 2019), wider eDNA-based monitoring schemes may rapidly become needed. In fact, one of the first eDNA-based tools for environmental surveillance of a snail vector, *Bulinus truncatus*, was developed in response to a local outbreak of urogenital schistosomiasis in Corsica, France, outside what is normally considered to be endemic areas (Boissier et al., 2015; Mulero et al., 2019). Likewise, methods for eDNA detection of the invasive *Aedes* mosquito, has recently been developed and tested in Southern Europe (Schneider et al., 2016). The ease with which eDNA samples can be collected, could for instance be combined with citizen science approaches (Biggs et al., 2015; Madden et al., 2016; Tøttrup et al., 2021). This would enable surveillance efforts for these emerging parasites and invasive vectors to be scaled up

substantially, both in terms of geographical and temporal coverage.

To fully utilize the potential of eDNA in parasitologists' 'molecular diagnostic toolbox', and for eDNA-based methods to become operational in LMIC settings, further methodological developments in eDNA may however be required. Luckily the broader field of eDNA-based monitoring is burgeoning, with new innovations and exciting areas for future research. Below we outline a number of research topics that, with continuous development, holds exciting potentials for future eDNA-based applications in parasitology and for improved monitoring and control of parasitic diseases.

5.1. eDNA for rapid, on-site and affordable detection and monitoring

On-site (field-based), real-time monitoring is often critical for timely and informed health interventions and decisions, when monitoring disease-causing parasites and vectors. To further extend the applicability of eDNA for such purposes, especially in remote, rural areas or in resource scarce environments, development of on-site, portable and low-cost technology for pathogen or vector detection using eDNA is needed. Another challenge that currently hinders further application of eDNA in LMIC settings, is the fact that eDNA-based methods requires specialized, state-of-art equipment and laboratory facilities. Despite the recent improvements due to COVID-19 (Gebremeskel et al., 2021)., such equipment and facilities are typically underrepresented in LMIC countries (Hamdi et al., 2021). This may also be part of the reason for the low number of eDNA studies identified in LMICs and in particular in Africa (Fig. 5b). Low-cost options for eDNA monitoring are therefore needed for the countries that may lack the necessary infrastructure (Ibaba and Gubba, 2020).

A way forward may be to take eDNA monitoring "out of the lab" and into the actual environment in which parasitic disease transmission takes place and where the samples are collected. This will reduce transportation time of eDNA samples to suitable laboratories, possibly hindering rapid detection of problematic parasites or vectors in the environment (Nguyen et al., 2018). Recent development in utilizing portable PCR machines (i.e. Biomeme), possibly connected to a smartphone (Priye and Ugaz, 2017; Thomas et al., 2020), and sequencers (i.e. MinION Oxford) offers great potential for rapid on-site detection of parasites and vectors species (Nguyen et al., 2018; Zowawi et al., 2021). These rapid tests could be used to help identify specific parasites and vectors in real-time at high spatial resolution, enabling fine scale precision mapping and more targeted interventions and control.

Another promising recent development in this context, is the combining of eDNA-based species detection with isothermal DNA amplification (amplification at a single temperature, e.g. 37 °C), i.e. Loop mediated amplification (LAMP). This has already been developed for detection of *Galba truncatula* snails, vector for several trematode species (Davis et al., 2020). Furthermore, this can be coupled to CRISPR/Cas technology (gene editing tool adapted for detection at a single temperature such as 37 °C) (Williams et al., 2021, 2019). This approach has proven efficient in differentiating closely related species and thus could be adapted to detection of disease causing parasites or other relevant parasite species such as invasive species. For several parasite and vector species, the first step of developing isothermal DNA amplification methods to be used directly in the field or in resource limited locations has already been done, i.e. *Fasciola hepatica* and *F. gigantica* (Tran et al., 2020), *Toxoplasma gondii* (Lass et al., 2017), *Dracunculus medinensis* (Boonham et al., 2020), *Plasmodium* sp. (Cook et al., 2015), *Cryptosporidium* sp. (Fallahi et al., 2018), and *Schistosoma* sp. (Lodh et al., 2017) as well as for the Asian schistosome intermediate host snail, *Oncomelania hupensis* (Tong et al., 2015). There is currently a fast development and adaptation of CRISPR/Cas for parasite detection in final hosts (Lee et al., 2020; Yu et al., 2021) suggesting that on-site detection of parasite transmission might become a reality in a not so distant future.

5.2. iDNA: Detection of parasite wildlife reservoirs

A number of the vector borne parasitic diseases are capable of infecting multiple definitive hosts, including domestic and wild animals. As a result, the parasites can be sustained in wild animal reservoirs complicating disease control and eventually jeopardizing elimination (Boonham et al., 2020; Catalano et al., 2020). This is problematic since the role and presence of wild animal reservoirs in sustaining transmission in an area often is unknown, and the traditional methods for handling, trapping or sampling wild animals to detect parasite infections can be challenging.

In conservation, eDNA methods are increasingly supplementing the traditional, often cumbersome, ways of monitoring vertebrate biodiversity in natural systems in specific eco-systems (Mena et al., 2021). In parasitology, the application of eDNA methods for detection of the potential wildlife reservoirs could be a valuable addition to the assessment of human infections, not least in areas where elimination is a target. For example, the detection of eDNA from potential wildlife reservoir species such as rodents and monkeys in samples from water bodies, could be important in the endgame of reaching elimination of schistosomiasis (Catalano et al., 2020; World Health Organization, 2022).

The novel development in this field is the use of iDNA (see Box 1), as mentioned in section 2, where examination of blood-sucking or biting invertebrates for DNA traces of the vertebrates they feed on/from (Calvignac-Spencer et al., 2013; Lynggaard et al., 2019). Several invertebrates have been used for vertebrate biodiversity detection, i.e. ticks (Gariepy et al., 2012), mosquitoes (Kocher et al., 2017), and leeches (Schnell et al., 2018). However, from our literature search only one study have analyzed sub-tropical, urban mosquitoes (*Aedes*) specifically to detect vertebrate reservoir hosts of mosquito borne parasites (Hopken et al., 2021). Further development here would be to use bulk insect samples (Lynggaard et al., 2019), instead of single specimens, to reduce costs and increase spatiotemporal coverage.

6. Concluding remarks

The ability to screen environments for the presence of parasites as well as vectors, intermediate and definitive hosts, has potential for diverse applications in human health, animal welfare, freshwater fisheries, coastal aquaculture, conservation, and ecosystem health. Yet, applications of eDNA technology for human and veterinary parasitology and disease surveillance, are still in their infancy (Fig. 4). This review finds that most studies so far has focused on using eDNA for detection of trematodes and intermediate host snails. Most studies have been conducted in Europe despite the obvious potential in developing countries with the highest parasite burdens. Although there are limitations, such as the inability of eDNA to distinguish between different life stages of the same species, eDNA methods have a large un-filled potential to be explored. This is for instance the timely monitoring of vectors appearing in low numbers in the environment, i.e. at the start of an "invasion", as climate change and environmental change are changing their geographical distributions. As many of the major human parasitic diseases in low- and middle-income countries are moving from control towards elimination, there is an increased need for more sensitive, cost-effective monitoring of environmental transmission, and eDNA is a valuable add-on to the parasitologist' tool-box in the future.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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