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Review

Exploring the role of circulating microRNAs as potential diagnostic markers in Mycobacterium paratuberculosis

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Abstract

Extracellular vesicles (EVs) are cell-derived and play a notable role in the development of chronic diseases and can be used as biomarkers as they transport microRNAs (miRNA). Existing research has found that most miRNA functions are carried out via intercellular transmission of EVs, which can protect and sort miRNAs. Early detection of disease is crucial for controlling the spread of the disease and improving livestock prognosis. miRNAs play a promising role as circulating biomarkers for early identification of disease. miRNA expressed in paratuberculosis infection has been identified using a variety of samples, including tissue, serum, whole blood, and macrophages. Key findings state that bta-miR-150 and bta-miR-1246 were observed in macrophages, whereas miR-29a and miR-92b were detected in serum and intestinal tissue. such as miR-143 and miR-485 underscore their potential as diagnostic and prognostic biomarkers in different stages of the disease. Studies on personalized medicine involve the detection of biomarker molecules such as miRNA and modulating therapy depending on the patient's severity of the disease, applying techniques like Northern blotting, qPCR, microarrays, and NGS. The current review assesses the potential use of circulating microRNAs as biomarkers for early and accurate diagnosis of *Mycobacterium avium* subsp. *paratuberculosis* infection, where the microRNAs could even serve as a point-of-care test.

Keywords

Diagnostic biomarkers, Paratuberculosis, point-of-care test, Infectious disease, miRNA

Introduction

Extracellular vesicles (EVs) are membrane-enclosed entities found in almost all human body fluids, ranging in size from 50 nm to 2 µm. They are rich in bioactive components like protein, nucleic acid, and lipids, which can be used to study the onset and prognosis of various chronic diseases. MicroRNAs (miRNAs) are short noncoding RNAs produced by all eukaryotic cells and are found in body fluids such as plasma, urine, saliva, bronchoalveolar lavage fluid, amniotic fluid, and semen (Xu et al., 2022). Circulating miRNAs remain stable in bodily fluids and can be protected by binding to argonaute proteins, high-density lipoprotein (HDL), or encapsulation in EVs. MicroRNAs regulate protein expression by binding to mRNA. EVs from diseased sources have distinct miRNA expression profiles that can reflect disease presence in early stages, dynamic disease development in late stages, disease prognosis, and drug resistance (Xu et al., 2022). Johne's disease (JD), often known as granulomatous enteritis or bovine paratuberculosis (paraTB) affects non-ruminants and ruminants globally. This disease is caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP) and is primarily evidenced by weight loss, diarrhea and a reduction in milk production (Badia-Bringue et al., 2024; Barad et al., 2014; Rawat et al., 2014). JD causes huge losses in production and economy, and it also poses a serious threat to animal welfare because it causes wasting and starvation in animals (Bush et al., 2006; Fernandez-Silva et al., 2014; Wright et al., 2023). Across geographical regions and production systems, there is well-documented epidemiological variability in the prevalence of MAP among livestock species, especially goats and sheep. The prevalence of MAP is about 28.2% in India, and the highest positivity rate is 42.7% in

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goats, followed by sheep (35.7%), cattle (24.1%), and buffaloes (20.6%). As a result of factors like husbandry practices, breed susceptibility, levels of biosecurity, and access to diagnostic testing, the northern regions of India report a notably higher prevalence of MAP than the southern states. The "Indian Bison type," which is the predominant biotype in India, also suggests that there may be interspecies transmission (Whittington et al., 2019; Chaubey et al., 2019). Despite the fact that such epidemiological patterns have typically been discussed in theoretical terms, they present a strong argument for the use of miRNA-based diagnostics in practice. Circulating miRNAs, such as miR-29a and miR-92b, which are detectable in serum, may be useful as early markers of subclinical MAP infection in high-prevalence areas like northern India (He et al., 2024; Wang et al., 2020). This is especially important for small-scale goat farms with inadequate diagnostic facilities. Similarly, research on sheep in southern Europe has shown that their miRNA changes in response to MAP exposure are stage-dependent, confirming their use in early disease surveillance. Targeting markers like miR-143 and bta-miR-150, miRNA assays based on fecal or blood samples may aid in routine monitoring and disease progression assessment in arid regions of Australia, where extensive sheep grazing systems are prevalent (Liang et al., 2016; Wang et al., 2019).

These previous studies highlighted the practical application of miRNA diagnostics in tackling the variable epidemiology of MAP and provide a path forward for incorporating molecular tools into herd-level disease control plans. There are some scientific reports attributing MAP to human autoimmune disorders viz., inflammatory bowel disease, Alzheimer's disease, Type-1 Diabetes, autoimmune thyroiditis, multiple sclerosis, and rheumatoid arthritis (Badia-Bringue et al., 2024; Juste et al., 2009; Pierce et al., 2018; Dow et al., 2021). The current diagnosis of a disease is inadequate due to its low sensitivity, time-consuming nature, and intermittent shedding of bacteria. This results in delayed detection and treatment, promoting disease development and transmission, and necessitating the use of PCR for specificity (Gupta et al., 2019).

17-25 nucleotide miRNAs are a class of small non-coding RNAs are conserved throughout the species. Numerous studies have demonstrated that miRNAs are expressed in various cell types and tissues (Wang et al., 2016). Serum, plasma, and other bodily fluids contain miRNAs that are well conserved and shielded from RNases' endogenous action. Initially, patients with diffuse large B-cell lymphoma were examined using circulating miRNAs as biomarkers in their serum (Mitchell et al., 2008). miRNAs have gained interest as potential diagnostic biomarkers for various disorders, including John's disease. Researchers have extracted microRNA from cattle's serum, blood, tissues, and feces to discover diagnostic biomarkers (Choi et al., 2021). There have been reports of extensive miRNA repertoires in the serum of MAP-infected cattle (Farrell et al., 2015; Liang et al., 2016). All of these findings point to the possibility that the amount of circulating miRNA varies with infection and may even be correlated with infection severity (Gupta et al., 2018). Their ability to improve early MAP detection and circumvent current diagnostic limitations makes them a promising research area. The review discusses the role of miRNAs in paratuberculosis diagnosis and their potential in both clinical and point-of-care applications.

Advantages of miRNA Biomarkers in the Detection of paraTB infection

Phase I: Detection of Latent Infections

Animals can be identified as having either a latent or a patent infection if they have MAP infection (Bastida et al., 2011). Severe forms of the disease show high infection frequency and patent granulomatous lesions. They progress from the latent forms with low or moderate infection frequency, characterized by focal histological lesions in intestinal tissues. A patent form often correlates with clinical conditions, while a latent form indicates a silent PARATB (Vázquez et al., 2020). Currently, diagnostic methods for latent infection detection have low sensitivity and specificity due to low bacteria excretion and low specific antibody titers in animals. However, any miRNA has not been identified from latent phase of paraTB infection till date. miRNAs serve as prognostic markers to determine animals at increased risk for the disease and thus allow for more specific and more efficacious preventive treatments (Tribolet et al., 2020).

Phase II: Early Detection

Early identification of infectious diseases is often crucial for controlling its spread and improving the prognosis of the animal. As the condition worsens, treatment options often become increasingly limited (Gupta et al., 2018). In MAP infection, early intervention of disease is crucial because animals with infection may remain asymptomatic for two to five years (Tribolet et al., 2020). Diagnosis of Johne's disease is usually done by fecal culture, but the technique is

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labor-intensive and of low sensitivity (Sharma et al., 2020). PCR detection is the most accurate because it can detect subclinical and clinical animals that are shedding MAP (Giese et al., 2000). PCR is more sensitive and specific than fecal culture for detecting MAP and, unlike immune-based assays, is not dependent on either Th1/IFN-γ or Th2/antibody responses (Begg et al., 2011; Chaitanya et al., 2022). Jain et al., (2021), when compared rELISA to fecal PCR it found more sensitive and specific than fecal PCR. As a result, it can be regarded as a useful tool for diagnosing paratuberculosis, overcoming the drawbacks of current techniques, and opening the door to improved disease treatment in cattle. Metcalf, (2024), explains the promising role of microRNAs (miRNAs) play as circulating biomarkers for early identification of hard-to-detect malignancies (Metcalf et al., 2024). This emphasizes how sensitive and precise diagnostic signatures can be obtained using miRNAs. In comparison to traditional diagnosis methods for *Mycobacterium avium* subspecies *paratuberculosis* (MAP) like culture and PCR, miRNA-based diagnosis has a number of clear advantages and future promise. Culture, though regarded as the reference standard, is notoriously time-consuming-taking anywhere up to 16 weeks to generate a result-and is low in sensitivity, particularly in subclinical infections. PCR-based approaches, as faster, also have the limitations of picking up DNA from viable and dead organisms, making for false-positive problems and the need for quality DNA and facilities. In comparison, miRNA biomarkers that remain stable in the body fluids and are recoverable through non-invasive means are capable of presenting early host-pathogen interaction as well as immunity, making detection potentially possible sooner Table 1. In addition, recent developments in portable qPCR platforms and point-of-care technologies have increased the viability of using miRNA-based assays in field settings (Choi et al., 2021; Gao et al., 2025). With initial setup costs possibly being higher, however, once validated, these procedures might provide scalable, multiplexed, and high-throughput diagnostics with little sample preparation, constituting an appealing alternative for MAP detection in clinical as well as herd-level screening programs.

Diagnostic Method	Sensitivity	Specificity	Time to Result	Sample Type	Field Applicability	Quantitative Validation Available	Notes
Fecal Culture	Low (30–60%)	High (≥95%)	8–16 weeks	Feces	Low	Yes	Gold standard; slow; less effective for subclinical cases
CR (qPCR)	Moderate–High (60–90%)	High (85–98%)	1–2 days	Feces, tissue, milk	Moderate	Yes	Detects DNA; possible false positives from dead bacteria
Serology (ELISA)	Low–Moderate (30–50%)	Moderate (70–90%)	Hours–1 day	Serum	High	Yes	Inexpensive, but poor early detection
miRNA-based	Not well defined	Not well defined	1–2 days (lab-based); <1h (PoC prototype)	Serum, plasma, tissue	High (potentially)	No	Promising for early detection; lacks standardization and independent validatio

Table I. Comparative Overview of Diagnostic Methods for MAP Detection.

Phase III: Improved Pathogen Identification

When symptoms of diseases initially appear, they often lack specificity and provide little to no insight into the underlying cause (Tribolet et al., 2020). Due to the specificity of these bacterially encoded miRNAs, studies have shown that miRNAs are strongly produced during active infection and suggest that these molecules can be used as biomarkers for MAP infection (Table 2). A number of studies have implicated host miRNAs differentially expressed upon MAP infection. These miRNAs are speculated to modulate important immune pathways, such as inflammatory signaling, autophagy, and apoptosis, central to the survival and persistence of MAP. Table 2 presents miRNAs implicated in MAP pathogenesis, along with their biological function. For instance, miR-146a regulates the innate immune response by inhibiting TRAF6 and IRAK1, critical mediators in the NF-κB signaling pathway, thus potentially dampening effective immunity and facilitating MAP persistence.Various samples, including tissue, serum, whole blood, and macrophages, have been used to identify the miRNA expressed in paratuberculosis infection (Table 2 and Table 3). These findings support the theory that miRNA biomarkers can distinguish between several causative agents in addition to differentiating infected from uninfected individuals. Additional information about the possible use of miRNAs in the diagnosis of infectious diseases may be found in further research (Ojha et al., 2019).

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S. No	Sample Type	microRNAs	Result	Function of miRNAs in bacterial infection	References
1	Tissues	miR-144, miR-19a, miR32, miR-139, miR-2425-3p	miR-2425-3p, miR-139; Upregulated miR32, miR-19a, miR-144 were Downregulated.	miR-144: target 4a (ATG4a) gene to inhibit autophagy.	(Badia-Bringué *et al.,* 2024; Guo *et al.,* 2017)
2	Serum	miR-205, miR-432 miR-29a, miR-92b	miR-205 was found to be upregulated miR-432 was found to be down-regulated Both are upregulated	miR-29a: Regulating the expression of interferon-gamma (IFN-γ)	(Farrell *et al.,* 2015; Vosgha al., 2014; Shaughnessy *et al.,* 2015; He *et al.,* 2024)
3	Whole blood	bta-mir-19b, bta-mir-19b-2, bta-mir-1271, bta-mir-7857, and miRNA 14_7917	bta-mir-7857, miRNA 14_7917: Upregulated bta-mir-19b, bta-mir-19b-2, bta-mir-1271: Downregulated	miRNA 14_7917: regulating immune-related gene expression. mir-1271: cell membrane for transport, volume regulation, and membrane potential stabilization.	(Malvisi *et al.,* 2016)
4	Intestinal tissues	miR-143	miR-143: Upregulated	Not specific	(Liang *et al.,* 2016; Vacante *et al.,* 2019)
5	Monocyte-derived macrophages (MDMs)	bta-miR-1246, bta-miR-132, bta-miR-122, bta-miR-150, bta-miR-92b, bta-miR-1343-3p, bta-miR-1306	bta-miR-1246 , bta-miR-132, bta-miR-122, bta-miR-2484: Upregulated bta-miR-92b, bta-miR-1343-3p, bta-miR-1306: Downregulated	bta-miR-150: suppresses cell apoptosis by targeting PDCD4 gene bta-miR-1246: Regulating the NF-kappa B signaling pathway for immune responses and inflammation. bta-miR-132: regulates the immune response by targeting p300 of various signaling pathways.	(Wang *et al.,* 2020; Wang *et al.,* 2019; Hussain *et al.,* 2018; Pu *et al.,* 2020; Guo *et al.,* 2020)
6	Jejunal lymph nodes (JELN)	bta-miR331-5p, bta-miR-2285bk, miR-146a and miR-146b	bta-miR331-5p: Upregulated bta-miR-2285bk: Downregulated	Not specific	(Guo *et al.,* 2020; Nahand *et al.,* 2020; Li *et al.,* 2021; Ibeagha-Awemu *et al.,* 2019)
7	Jejunal intestine	bta-miR-485, bta-miR-451, miR-147 and miR-199a-5p	bta-miR-485; Upregulated bta-miR-451; Downregulated	miR-147 and miR-199a-5p: Associated with glucose metabolism.	(Guo *et al.,* 2020; Nahand *et al.,* 2020; Li *et al.,* 2021; Ibeagha-Awemu *et al.,* 2019)
8	Macrophages	miR-27a-3p, miR-24	miR-27a-3p: Downregulated	Not specific	(Zhao *et al.,* 2016; Hussain *et al.,* 2018)
9	Serum	miR-1976, miR-873-3p, miR-520f-3p & miR-126-3p	miR-1976 & miR-873-3p: Upregulated miR-520f-3p & miR-126-3p: Downregulated	miR-1976: Triggered apoptosis, and depressed cell viability in SUM-1315 and MDA-MB-231. miR-126-3p: Activates mTOR signaling, to stimulate & enhance macrophage function for an effective immune response against the infection.	(Gupta *et al.,*2018; wang *et al.,*Zou *et al.,* 2021; Chen *et al.,* 2020; Ichikawa *et al.,* 2020; Martinez-Arroyo *et al.,* 2023)
10	HumanTHP-1 cell line	miR-21	miR-21; Upregulated	miR-21: Involves in the autophagy and apoptosis regulation.	(Wright *et al.,* 2021; Zhao *et al.,* 2019; Mostoufi-Afshar *et al.,* 2018; Jenike *et al.,* 2021)
11	Serum	bta-miR-363, bta-miR-374b, bta-miR-2887	bta-miR-363, bta-miR-374b, bta-miR-2887; Upregulated bta-miR-147, bta-miR-196a, bta-miR-346, bta-miR-655, bta-miR-2426; Downregulated	Not specific	(Zhang *et al.,* 2020; Xia *et al.,* 2019; Chengcheng *et al.,* 2024; Li *et al.,* 2020)
12	Peripharal blood samples (PBMC)	bta-miR − 2484, bta-miR- 1434-5p	bta-miR − 2484, bta-miR- 1434-5p; Upregulation	bta-miR- 1434-5p: Control the expression of CXCL5, CXCL8, and CXCL9 in inflammatory disease.	(Singh *et al.,* 2020; Lv *et al.,* 2021)
13	Faecal	hsa-miR-92a-3p, miR-501-5p and hsa-miR-658	hsa-miR-92a-3p; Upregulated miR-501-5p and hsa-miR-658: Downregualted	miR-92a-3p: Regulates PTEN to enhance the proliferation, migration, and invasion of esophageal squamous cell carcinoma. miR-501-5p: Downregulates LPAR1 in gastric cancer to increases the proliferation and migration of cells.	(Li *et al.,* 2019; Shaughnessy *et al.,* 2020)

Table II. Identification of potential miRNAs biomarkers in different clinical samples.

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S. No	Function	miRNAs	References
1	Modulating inflammatory responses	miRNA-144, miR-32miR-139miR-19amiR-144bta-miR-132bta-miR-2285bk	(Badia-Bringué *et al.,* 2024; Wang *et al.,* 2019)
2	Innate immunity	bta-mir-19b-2bta-mir-19bmiR-1271bta-miR-2484	(Singh *et al.,* 2020; Malvisi *et al.,* 2016)
3	Cell mediated immunity	bta-miR-331-5p	(Ibeagha-Awemu *et al.,* 2019)
4	Apoptosis	miR-27a-3p	(Hussain *et al.,* 2018)
5	Autophagy	miR-21	(Zhao *et al.,* 2019)

Table III. Summarizing the role of miRNAs in the pathogenesis of paratuberculosis.

Phase IV: Personalized Medicine

Personalized medicine, precision medicine, or diagnostics, aims to tailor treatment to individual patients' disease severity and treatment effectiveness, using biomarkers for efficient decision-making. In the context of personalized medicine, "biomarkers" typically refer to a person's genotype; however, additional diagnostic or prognostic markers, such as miRNAs, are also being considered in this context (Tribolet et al., 2020). This method, like many other aspects of medicine, started off in oncology and is now making its way to other fields, such as infectious diseases (Liu et al., 2014). Authors defined various miRNAs enlisted in Table 3 that distinguish upregulated and downregulated-miRNAs and provide essential information regarding the animals's reaction to infection, assisting in the identification of animals at high risk for infection and directing medical decisions. These findings validate the use of this miRNA to track the development of infection, forecast the effectiveness of treatment, and improve prognostic precision. While many studies describe the use of miRNA biomarkers for diagnostic purposes (Wang et al., 2020; Shaughnessy et al., 2020; Zhao et al., 2016), the research previously stated shows that miRNAs can also be used as companion diagnostics to advise optimal therapeutic choices, quantify the effectiveness of therapy, and predict disease outcome and host responses.

miRNAs as potential biomarkers of paratuberculosis

Numerous findings claim that when a host becomes infected with MAP, miRNAs have the ability to control the expression of genes involved in either the innate or adaptive immune pathways (Malvisi et al., 2016; Liang et al., 2016). Thus, certain miRNAs have the potential to be employed as a diagnostic or prognostic biomarker for paratuberculosis due to their relationship with any immunological response (Pattnaik et al., 2022). In this review, we enlist a few miRNAs can we used as potential diagnostic markers (Table 2). The selected miRNAs can control the activation of immune cells. Pathological mechanisms implicated in tuberculosis infection include autophagy and apoptosis. Specific miRNA molecules display certain advantages and disadvantages as biomarkers concerning infection of *Mycobacterium avium* subsp. *paratuberculosis* (MAP). For example, bta-miR-150 and bta-miR-1246 are highly specific in that they regulate immune responses, rendering them reliably useful as diagnostic markers for MAP (Wang et al., 2019). In contrast, miR-21 and miR-146a are more related to general inflammation and are thus implicated in potential false positives in infections other than MAP (Jenike et al., 2021; Nahandet al., 2020).Stabilities of these miRNAs in bodily fluids are, therefore, another crucial element; bta-miR-92b and miR-29a can easily be detected in serum and whole blood (Guo et al.,2020; He et al., 2024), suggesting they can be suitable for non-invasive diagnostics; however, bta-miR-331-5p and miR-2285bk found in tissue samples would be impractical in routine screening (Ibeagha-Awemu et al., 2019). Early detection has been attributed to the advantages of miRNAs such as miR-19b and miR-1271 that show altered expression with the onset of MAP infection, favourably positioning them in diagnostics over classical means (Malvisi et al., 2016). Their minor differences in expression, however, necessitate sensitive detection technologies-facilitating methods such as qPCR or next-generation sequencing-, which may not be extensively available. Independent from being a valuable marker, miRNAs, like bta-miR-485 and miR-199a-5p, will also influence the progression of the disease by affecting various metabolic and immune pathways; hence, this represents a prognostic value (Ibeagha-Awemu et al., 2019). Nevertheless, the highly variable expression of miRNAs at designated disease stages further complicates their standardization for diagnostic purposes.

More recent research has contrasted the diagnostic performance of miRNA-based tests for the quantitative detection of *Mycobacterium avium* subsp. *paratuberculosis* (MAP). The following, specifically: Gupta et al. (2018) found 87.5% sensitivity and 91.3% specificity using serum miRNAs in infected cattle. Choi et al. (2021) showed 80% to 85% diagnostic sensitivity. Farrell et al. (2015) showed 75-88% sensitivity in early and subclinical infections. miRNA-based tests are promising for diagnosing *Mycobacterium avium* subsp. *paratuberculosis* (MAP) infections, with sensitivity

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ranging from 60-90% and specificity to fecal culture (30-60%). However, large-scale studies are needed to validate these tests.

Furthermore, while the serological detection of other miRNAs, like miR-205 and miR-432, is attractive for point-of-care testing, detection methods that are both affordable and feasible for field use are still being developed, therefore hindering their immediate clinical application (Vosghaet al., 2014; Farell et al., 2015). Overall, while the application of miRNAs in paratuberculosis diagnostics remains a potent one, its implementation requires validation to optimize specificity, accessibility, and feasibility of detection in practical settings. Tables 2 and 3 show that miRNAs act as biomarkers for the detection of MAP but also have mechanistic insights towards the pathogenesis of disease. Given their involvement in immune modulation, host-pathogen interactions, and cellular responses, they could be useful in complementary improvements in the diagnostics of paratuberculosis. Further, research is needed to evaluate specificity, optimize detection methods, and translate them into field-applicable diagnostic tools.

Current miRNA Detection Platform

Next generation sequencing (NGS), qPCR, microarrays, and northern blotting are some of the conventional methods for finding miRNAs. Northern blotting and microarrays have become less popular due to several drawbacks, such as low sensitivity or specificity and increased total RNA input requirements (sometimes several micrograms for northern blotting). Instead, modern miRNA biomarker studies typically employ qPCR and NGS (Figure 1).

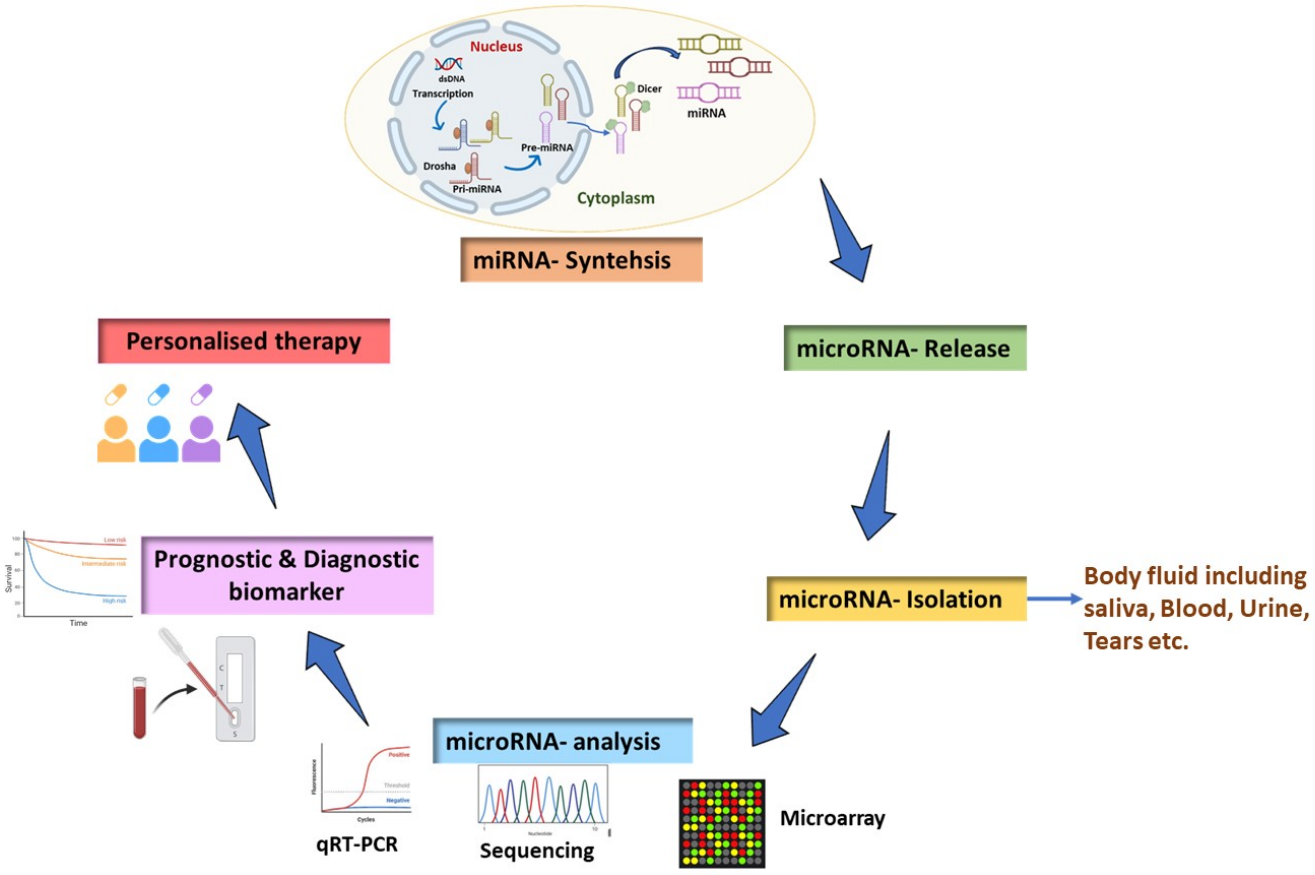

Figure 1. Diagrammatic representation of miRNA biogenesis and ways of secretion into bodily fluids. In the future, the condition can be targeted and treated individually using a clinical decision-making technique.

Quantitative real-time PCR (qRT-PCR) is reputed for its great analytical sensitivity and can detect microRNA concentrations as low as 1 to 10 femtomolar (fM) in biological samples when the target sequence is known and specific primers are used (Mitchell et al., 2008; Hardikar et al., 2014). It is very specific and suited well to targeted confirmatory research. On the other side, next-generation sequencing (NGS) allows for a true unbiased view of high-throughput profiling of miRNAs, including novel variants, up to Attomolar (aM) sensitivity owing to its deep sequencing capability and multiplexing (Kozomara et al., 2019; Vincent et al., 2017). Very often, qRT-PCR is sought in clinical settings or point-of-care scenarios for the reason of time and cost. With better dynamic range and resolution, NGS is suitable for discovery types of research in miRNA biomarker studies; hence, despite the cost and complexity, it is the

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choice. Mass spectrometry (MS) has been used recently by researchers to detect miRNAs (Kullolli et al., 2014). Various reviews discuss NGS and qPCR methods for miRNA detection (Hardikar et al., 2014; Vincent et al., 2017). miRNAs are reverse-transcribed to cDNA and then amplified using specific primers to be quantified via qPCR. After binding to the cDNA, a sequence-specific probe with a fluorescence and quencher is broken down by the DNA polymerase's endonuclease activity during amplification. When the fluorophore is released in this way, the fluorescence that results is measured (Wong et al., 2015). Even though next-generation sequencing also needs amplification and reverse transcription, it accomplishes it without the use of primers or probes that are particular to known miRNAs. Millions of short read sequences are produced, which can subsequently be mapped to a reference sequence like a genome or the miRNA sequence database (miRbase) or examined de novo (Kozomara et al., 2019). There are many NGS platforms with different proprietary chemicals available, but they are all large, costly (though costs are still dropping), labor-intensive, and necessitate intricate sample preparation, instrument operating, and data analysis processes. Despite ofthis, it is the preferred method for finding miRNA biomarkers initially, and qPCR assays are usually developed for additional validation (Tribolet et al., 2020). The transition of microRNA (miRNA)-based diagnostics from bench to bedside necessitates a systematic and multidisciplinary approach. The figure 2a depicts a comprehensive roadmap that addresses the key elements required for successful clinical implementation. This roadmap is built around four interconnected pillars: rigorous validation, regulatory alignment, technological integration, and cross-sector collaboration. The development of a reliable miRNA-based diagnostic test for *Mycobacterium avium* subspecies *paratuberculosis* (MAP) infection requires a structured approach, with agreed timelines (Figure 2b) for each critical step of research, validation, and implementation.

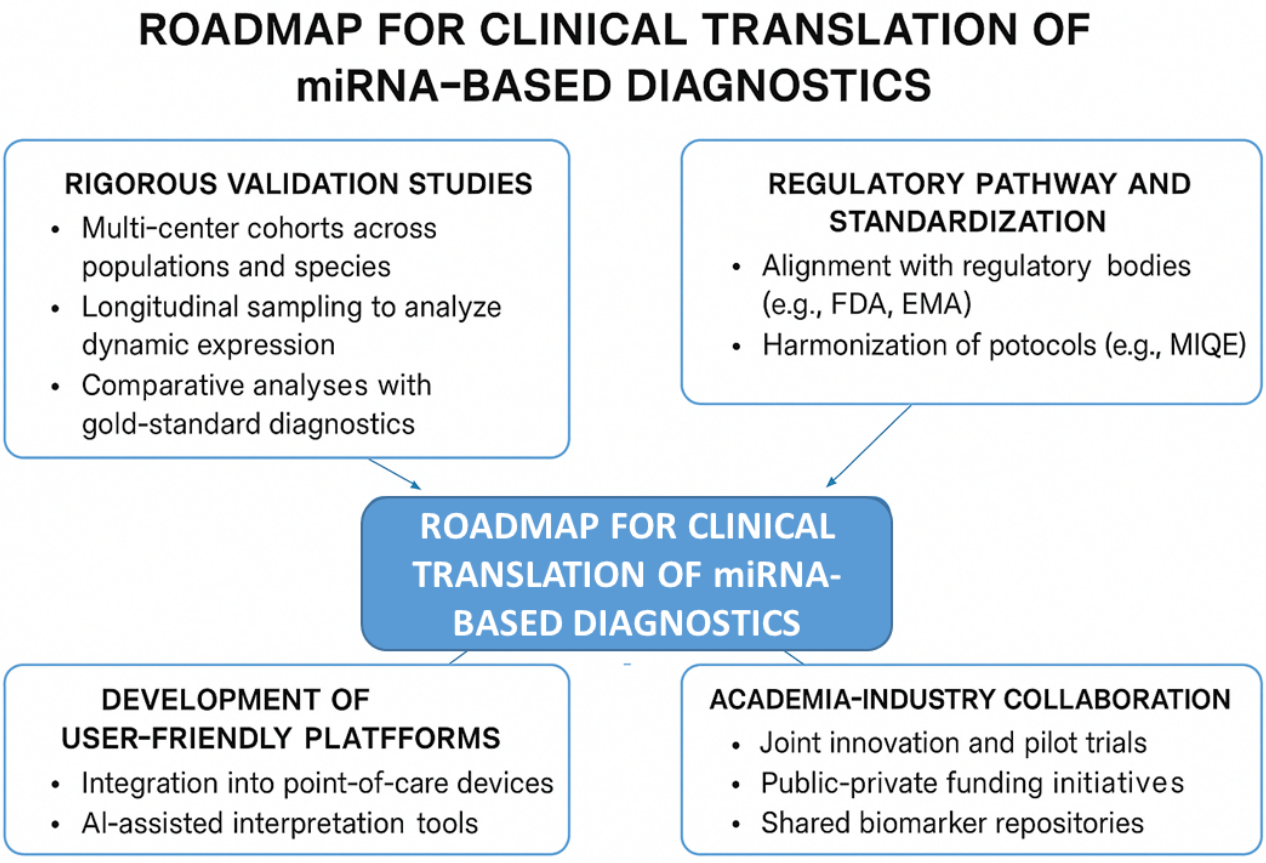

Figure 2a. Roadmap for clinical translation of miRNA based clinical diagnostic.

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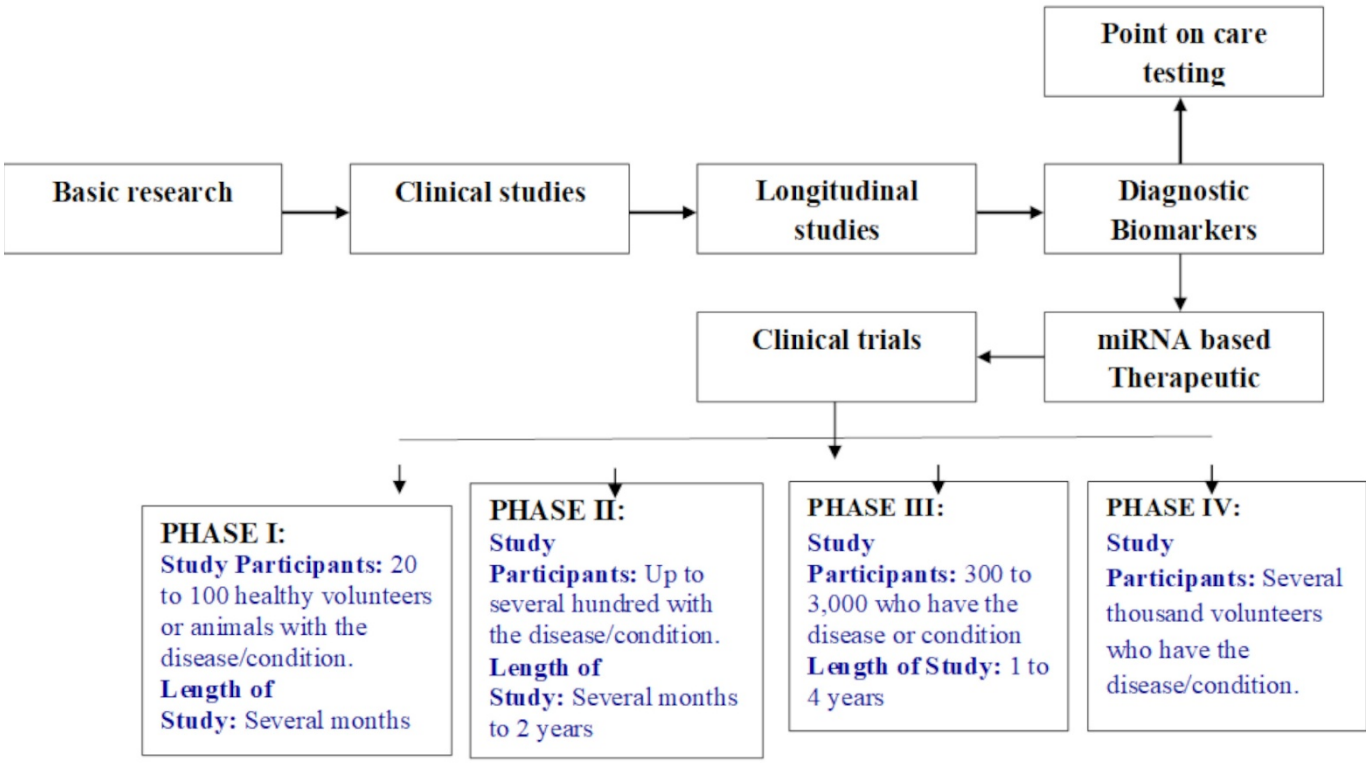

Figure 3b. Success Indicators and timeline for the clinical research. (https://www.fda.gov/patients/drug-development-process/step-3-clinical-research. accessed on 28 May 2025).

Novel and Promising Platforms for miRNA Detection

Emerging technologies for detecting miRNA biomarkers in infectious diseases focus on portability, robustness, speed, specificity, sensitivity, affordability, and ease of use. Transitioning from costly lab-based methods to point-of-care technologies is crucial for diagnosing and managing outbreaks in resource-limited settings (Liang et al., 2016).

Various technologies have been applied in miRNA detection for *Mycobacterium avium* subsp. *paratuberculosis* (MAP), which differ in sensitivity and specificity. Traditional methods like Northern blotting and microarrays may be qualitative but fail considerably in their sensitivity when large amounts of RNA need to be analyzed. The technique most used today is quantitative PCR (qPCR) because it is highly sensitive, but it requires that one know the target miRNAs beforehand. Next generation sequencing (NGS) also holds a lot of promise as far as sensitivity is concerned, but it is very costly and not practical for day-to-day diagnostics. PoC devices are those such as lateral flow assays and electrochemical biosensors, which provide fast and cheap alternatives but need more rigorous validations. Finally, to be able to improve MAP diagnostics, PoC devices will have to increase their capacity for detection and standardize it for better diagnostics. Advancements in in-lab miRNA detection technologies require integration into proof-of-concept devices and streamlined protocols for easy translation. The low concentration of miRNA in biofluids is a major obstacle to PoC device development, as miRNA in plasma is typically in the femtomolar range in healthy animals (Mitchell et 2008). Figure 3 provides an overview of various technologies. Lateral flow devices, or strip tests (Fig. 3A & 3C), are affordable, user-friendly, portable, and specific technologies used to accurately detect biomarkers at the point of care. Due to these advantages, numerous teams have developed strip-based biosensors for miRNA detection. Electrochemical biosensors have been effectively created by several research groups to detect miRNAs with high specificity and sensitivity (Fig. 3D) (Deng et al., 2017). Feng et al.,2017, developed a pH-dependent visual miRNA sensing method using hydrogen ions from netlike rolling circle amplification. They used cresol red, neutral red, and m-cresol purple pH indicators, observing a 100 femtomolar limit of detection for miR-21, which could be further analyzed using UV-visible spectroscopy (Fig. 3B).

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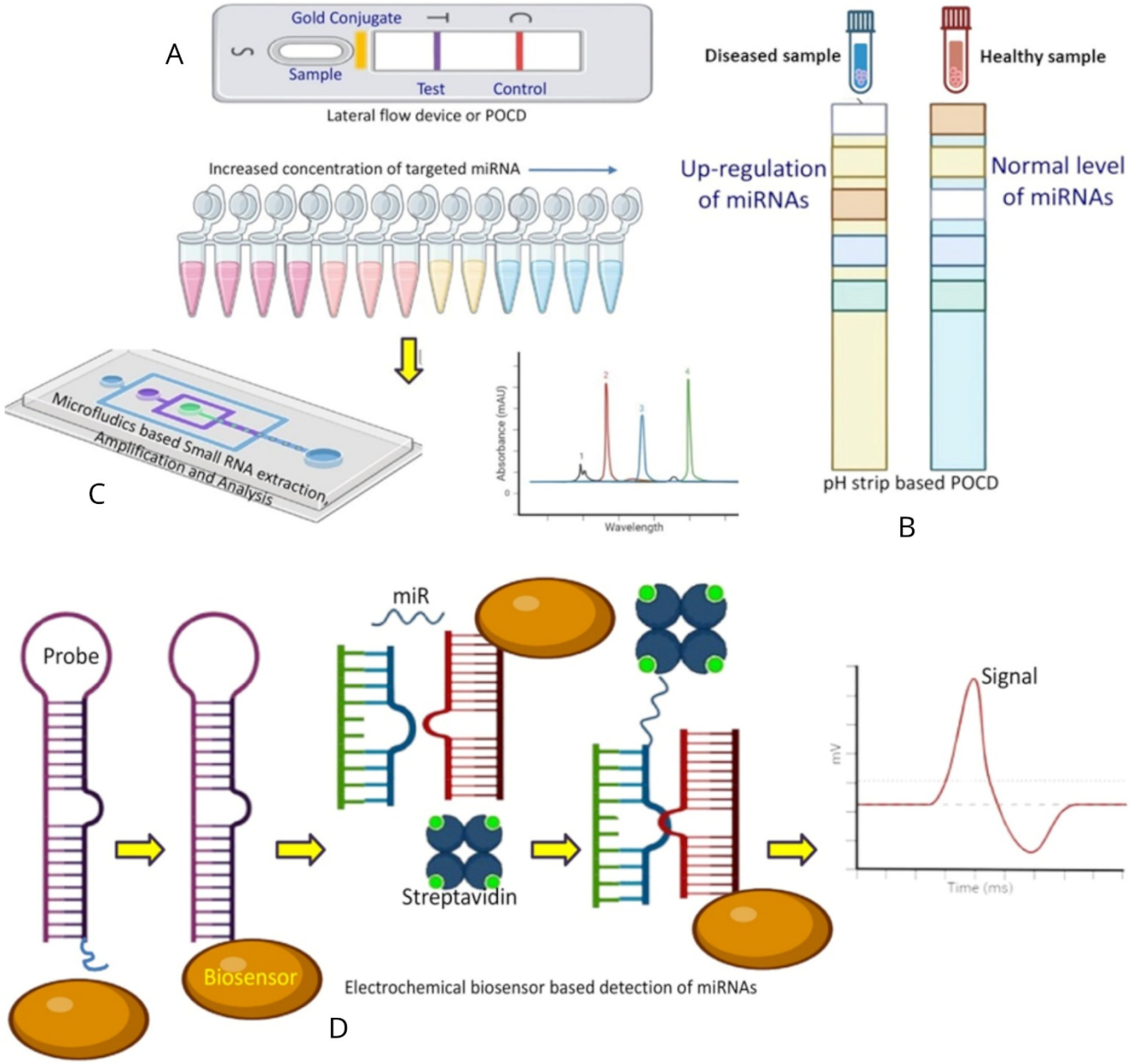

Figure 4. Point on care devices for detection of miRNA. (A) Lateral flow device (B) pH strip based POCD (C) Micropads for extraction and analysis of small RNA (D) Electrochemical.

Over the years, there have been notable advancements in the detection of miRNA using portable devices and visual detection techniques. These methods are beneficial in low-resource settings and enable rapid miRNA detection. Currently, most point-of-care testing (POCT) assays for miRNAs rely on capillary force meters, pressure meters, portable fluorometers, PGMs, and thermometers. However, these assays have only been able to detect extracted or synthesized miRNAs (Wang et al., 2023).

Some of the standardization issues that affect miRNAs as biomarkers for MAP are the discrepancies in sample collection processes as well as those in their processing, isolation, and storage methods, which have all been shown to have notable negative impacts on miRNA stability. The absence of consensus on the issue of normalization strategies and variation in data analysis as well as the problem of clinical validation adds to the plagues faced by miRNA in MAP diagnostics. Thus, it could be concluded that they are indeed required to improve the diagnostic use of miRNA in MAP.

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Challenges in Translating miRNA-Based Diagnostics into Clinical Practice

Lack of Standardized Procedures

One of the biggest issues is that there are no universal standards for sample collection (e.g., blood, milk, feces), small RNA-specific RNA extraction methods, and normalization controls for qRT-PCR or NGS. Many studies employ various internal controls (e.g., U6, miR-16, or spike-ins) (Sewer et al., 2014; Yang et al., 2022), and hence it is difficult to make inter-laboratory comparisons as well as meta-analyses. Various efforts are being made, including efforts such as the MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines that invite reporting on standardized methodology (Johnson et al., 2014). To ensure reliable and reproducible quantification of miRNA expression levels using RT-qPCR, appropriate endogenous controls (also known as housekeeping genes) must be carefully selected on following criteria: low variability (CV < 5%, SD < 1); have high expression (basemean ≥ 3000); and remain unchanged between sample groups (log2 fold change < 1) (Gupta et al., 2021).

Reproducibility and Analytical Validation

Reproducibility must be attained to gain regulatory approval and offer clinical reliability. Inconsistencies in the detection of miRNA on different platforms, sensitivity to minor alterations in input material, and between-sample variability all contribute to variable results (Hays et al., 2022). Current initiatives involve cross-platform comparison and validation within multicenter trials, with digital PCR being recognized as an acceptable method for absolute quantitation of miRNA (De Korne-Elenbaas et al., 2025).

Biological variability and confounding factors

miRNA expression is regulated by age, gender, circadian rhythm, nutrition, and comorbid conditions, and, therefore, disease-specific signatures may prove difficult to interpret (Virts et al., 2010; Llera-Oyola et al., 2024; DeLucas et al., 2024). Current research is oriented towards large-scale profiling in heterogeneous populations and machine learning model approaches to the detection of stable biomarkers.

Clinical verification and regulatory clearance

Regulatory bodies such as the FDA and EMA demand clinical utility, large validation cohorts, and cost. Only a handful of miRNA-based tests (mostly oncology) have been approved until now. Consortia for translation such as the Early Detection Research Network are filling this gap, and analogous programs in veterinary diagnostics. Integration with Clinical Workflow: Even certified miRNA tests need to be integrated into clinical use seamlessly, considering time-to-result, user-friendliness, and compatibility with installed base infrastructure. Ongoing research in point-of-care testing and microfluidic platforms aims to facilitate clinical adoption.

To achieve reliable inter-laboratory reproducibility and facilitate the clinical adoption of miRNA-based diagnostics, it is essential to establish harmonized operational protocols across laboratories. Standardization should begin with consistent sample collection procedures, including the use of EDTA-coated or serum-separating tubes, immediate centrifugation at 2,000 × g for 10 minutes at 4°C, and prompt storage of samples at –80°C to preserve RNA integrity. RNA extraction protocols must be unified using validated commercial kits with high miRNA recovery efficiency and the routine inclusion of synthetic spike-in controls, such as C. elegans miR-39, to monitor extraction quality (Sewer et al., 2014). For qRT-PCR assays, normalization strategies should rely on consensus endogenous controls like miR-16 or miR-191, selected based on low variability and expression stability, and evaluated using algorithms such as geNorm or NormFinder (Gupta et al., 2021; Hays et al., 2022). Calibration across platforms—qRT-PCR, NGS, and digital PCR—should involve the use of synthetic miRNA standards or reference curves to ensure quantification accuracy (Hays et al., 2022). Furthermore, data analysis should follow the MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines (Johnson et al., 2014), while metadata reporting can be standardized using MIMARKS criteria. To identify and minimize technical variability between laboratories, proficiency testing and ring trials should be implemented routinely. These measures will collectively reduce pre-analytical and analytical bias, enhancing the robustness, reproducibility, and translational utility of miRNA biomarkers in veterinary diagnostics (Gupta et al., 2021; Hays et al., 2022).

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Future prospects and Conclusions

miRNAs, small noncoding RNAs, are enriched in bodily fluids and differentially expressed in sick tissues, making them valuable for routine clinical diagnosis. Research has focused on identifying miRNA signatures indicative of various diseases, such as diabetes, cancer, viral infections, bacterial infections, particularly mycobacteria, nervous system disorders, cardiovascular disease, and muscular diseases (Wang et al., 2016; Parashar et al., 2022; Gilyazova et al., 2023) including paratuberculosis. miRNAs function as post-transcriptional regulators, controlling gene expression during development and disease progression. Healthy and ill individuals express miRNAs differently, and they are persistent in urine, saliva, and blood (Cheng et al.,2018). Research has shown that miRNAs can be useful non-invasive biomarkers for various diseases, both in terms of diagnosis and prognosis (Gupta et al., 2021). Numerous miRNAs have been described since their discovery. Even while research on the function of miRNAs in sick instances has advanced, many illnesses are still poorly understood because the area is still in its infancy. Finding these disease-specific molecules and incorporating their significance into the diagnosis, prognosis, and therapy plan, however, holds enormous promise. Numerous miRNAs are commonly altered in a variety of illnesses, including neurological disorders, sepsis, cancer, and cardiovascular disease (Sun et al., 2018; Nahand et al., 2019; Zhou et al., 2018), which is the leading cause of morbidity and mortality worldwide. New and improved techniques are required to recognize disorders linked to a higher death rate as soon as possible. miRNAs meet the necessary criteria to be included in this kind of procedure.

The future direction of research shall be directed towards standardization of protocols, enhancement of high-sensitivity detection methods (qPCR, NGS, biosensors) and integration of learning from machine exercise into biomarker validation for improvement of miRNA diagnostics strategies for MAP. Multi-center clinical studies should be conducted to ensure collective reproducibility across different populations, and such diagnostic technology development would make diagnosis possible for point-of-care applications. Development in these specific areas will make miRNAs sophisticatedly reliable and field-deployed tools for early detection of MAP infection.

In conclusion, current diagnostic methods for infectious diseases lack sufficient speed and accuracy. microRNAs (miRNAs) have emerged as promising biomarkers with high diagnostic and prognostic potential. Despite challenges in clinical translation, advancements in miRNA detection technologies and the development of commercial assays support their integration into diagnostic workflows. miRNAs are expected to play a pivotal role in personalized medicine by enabling precise patient stratification and targeted therapies. However, further research and clinical validation are necessary to establish their routine diagnostic use.

Ethical concerns and regulatory aspects of miRNA-based diagnostic tests in veterinary medicine

The development of miRNA-based diagnostics in veterinary medicine involves key ethical and regulatory considerations. Ethically, it is essential to ensure animal welfare during sample collection, obtain informed owner consent, and protect the privacy of genetic data. There are also concerns about equitable access, especially in low-resource settings. On the regulatory side, these diagnostics require rigorous validation for accuracy and reliability across species, adherence to biosafety and quality standards, and oversight by authorities like the OIE, EMA, or USDA. Species-specific guidelines and market authorization are crucial for clinical adoption.

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Conflicts of interest

Authors report no conflicts of interest in this work.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Author Contributions Statement

SA: Conceptualization, Methodology, Writing - Original Draft. SG: Data Curation, Investigation, Writing - Review & Editing, Supervision. SVS: Writing - Review & Editing, Supervision.

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