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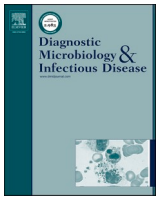

Microfluidic point-of-care testing for the detection of *Bordetella pertussis*: A mini-review

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ABSTRACT

Bordetella pertussis is a bacterial pathogen responsible for pertussis, which is a highly contagious respiratory disease. Despite the relatively high vaccination coverage, pertussis is considered a reemerging disease that necessitates enhanced strategies for identification, prevention, and control. The diagnosis of pertussis typically involves a combination of clinical evaluation, laboratory tests, and a thorough medical history. The current technologies for pertussis diagnosis have their own limitations, prompting the exploration of alternative diagnostic approaches that offer enhanced sensitivity, specificity, and speed. Microfluidic technology is considered a very promising tool for the diagnosis of infectious diseases, as it offers more rapid and accurate outputs. It allows point-of-care testing (POCT) at or near the patient site, which can be critical, especially for an outbreak or pandemic. In this paper, current pertussis diagnostic tools with their limitations were discussed, and microfluidic approaches for the diagnosis of pertussis were highlighted.

1. Introduction

Bordetella pertussis is an aerobic Gram-negative bacterium that demonstrates a rapid growth rate by its ability to adhere to the epithelial surfaces of the trachea and bronchi in the human respiratory system. It is the causative agent of pertussis, which is mainly characterized by severe coughing attacks and choking-like breaths, which can be lethal for infants and young children [1]. Pertussis is a highly contagious respiratory infection that affects all age groups, from infants to the elderly. Although it is commonly seen among infants and children, there has been a noticeable rise in incidence among adults and adolescents, leading to questions about the current vaccination schedule, especially for adults. It has also prompted the exploration of more efficient preventive measures, such as next-generation pertussis vaccines. The prevention of the disease can be achieved through vaccination with either whole-cell pertussis (DTwP) or acellular pertussis vaccines (DTaP), which are available only for use in infants and children under 7 years of age. For adolescents, adults, pregnant women, and children 7 years of age and older, a booster dose (Tdap) is also available [2]. Among pediatric vaccines, DTaP vaccine is more commonly used worldwide since the DTwP vaccine is too reactogenic. The use of DTaP vaccines is not without drawbacks, as there is often a resurgence of pertussis following the switch from DTwP to DTaP vaccines possibly leading to waning of immunity [3]. Despite routine vaccination for more than five decades, pertussis continues to be a significant respiratory illness that affects a wide range of age groups, from infants to adults. This emphasizes the criticality of an accurate diagnosis of pertussis in order to administer appropriate treatment and avoid further transmission of the disease.

The diagnosis of pertussis mainly relies on clinical manifestations and laboratory diagnostic methods, including culture, serology, and polymerase chain reaction (PCR) [4]. Despite their widespread use, these approaches have their own limitations, such as long incubation period of cultures, low sensitivity of serological tests, and need for experts for PCR-based studies [5]. Thus, there have been endeavors to enhance existing diagnostic tools and develop innovative methods in recent years. The use of microfluidic techniques in biotechnology for the detection of infectious diseases has emerged as a noteworthy and encouraging approach. This has resulted in the development of point-of-care testing (POCT), which enables rapid detection at site or next to the patient with the advantages of being simple, portable, automatic, and cost-effective [6]. With the outbreak of Corona Virus Disease 2019 (COVID-19), studies have been accelerated to implement POCT for the diagnosis of various diseases. Although there is limited availability of mature products for infectious diseases in the present market [7], the advancements in microfluidics, functional materials, and biosensing technologies would stimulate relevant researchers to

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seize the opportunities to make sample-to-result devices with great sensitivity and specificity.

This review centers on the discussion of existing pertussis diagnostic technologies, with a particular emphasis on the advancements made in the field of microfluidic POCT-based diagnosis of pertussis.

2. Unmasking pertussis: exploring current diagnostic approaches

2.1. Clinical diagnosis

The diagnosis of pertussis in a clinical setting is highly challenging due to the heterogeneity in the manifestation of the disease, particularly among adults and adolescents. The presence of mixed infections, the impact of immunization, and a low index of suspicion among physicians further complicate the diagnosis process [4]. In a typical disease course, the early stage of the infection (the catarrhal phase) is characterized by sneezing, non-specific coughs, and rhinorrhoea after an incubation period of 7-10 days. The subsequent phase, known as the paroxysmal phase, is described by whooping, vomiting, and spasmodic coughing attacks in unvaccinated children, along with leukocytosis and lymphocytosis. Apnea can be the only symptom in neonates and infants who have not received vaccinations. The last phase is the convalescent stage, during which the symptoms gradually decrease. However, it is important to note that a relapse of the disease may occur if another infection is encountered. This phase can last several months with a persistent cough, even after the most severe symptoms have subsided [8,9]. Based on the presenting symptoms, clinical case definitions for pertussis have been described by the World Health Organization (WHO) and the Centers for Disease Control and Prevention (CDC) (Table 1) [10,11]. In addition, the Global Pertussis Initiative has acknowledged the limitations of the existing definitions and has put up a set of more comprehensive criteria, which are categorized by age groups [12] (Table 1). Many low- and middle-income countries face significant limitations in terms of their access to diagnostic facilities and resources for confirming cases of pertussis. Consequently, these countries heavily rely on clinical diagnosis as their primary means of identification. Hence, it is critical to establish a robust clinical case definition for accurate detection of the disease [13]. Nevertheless, given the challenges associated with the clinical diagnosis, it is strongly recommended to acquire laboratory confirmation.

Table 1
Clinical case definition of pertussis for surveillance purposes according to WHO, CDC, and Global Pertussis Initiative.

Organization/Year	Clinical criteria
WHO, 2018 CDC, 2020	- Clinician suspicion of pertussis, or - A case with a cough lasting ≥ 2 weeks with at least one of the following symptoms (based on observation or parental report); • Paroxysms of coughing • Apnea (only in < 1 year of age) • Inspiratory whooping • Post-tussive vomiting, or vomiting without other apparent cause
Global Pertussis Initiative, 2012	0-3 months: cough and coryza with no or minimal fever plus: • Whoop or • Apnea or • Post-tussive emesi sor • Cyanosis or • Seizure • Pneumonia • Close exposure to a person with a prolonged cough illness 4 months – 9 years: Paroxysmal cough with no or minimal fever plus: • Whoop or • Apnea or • Post-tussive emesis • Seizure • Worsening of symptoms at night • Pneumonia • Close exposure to a person with a prolonged cough illness ≥ 10 years: Nonproductive, paroxysmal cough of ≥2 weeks without fever plus: • Whoop or • Apnea or • Sweating episodes between paroxysms • Post-tussive emesis • Worsening of symptoms at night

2.2. A brief look at laboratory diagnosis

Presently, diagnostic tools for the detection of pertussis encompass the utilization of culture techniques to isolate the etiological agent, PCR to identify pathogen-specific genes, and detection of the immune response to the virulence determinants. While PCR and culture exhibit the highest sensitivity within the initial two-week period of the disease, serological tests can be performed at a later stage compared to PCR and culture (Fig. 1). Specimen collection and transportation of the samples influence the sensitivity of culture and PCR. Nasopharyngeal swabs should be used to obtain a specimen from the surface of ciliated epithelial cells of the upper respiratory tract, as *B. pertussis* exhibits a preference for colonizing these specific cells. After the collection of samples, they should be promptly plated or immediately placed into a suitable transport medium, and thereafter plated within a 24-hour timeframe [14].

2.2.1. Culture-based method

The CDC asserts that culture remains the definitive method for diagnosing pertussis, despite its lengthy incubation period of up to ten days [14]. This approach is considered the gold standard due to its ability to provide a specificity of 100% in identifying the presence of pertussis. Moreover, the culture of the organism is also valuable since the isolates can be used to evaluate antimicrobial susceptibility, identify strain types, and track genetic changes [10]. It is most effective during the first two weeks of the disease following cough onset, as viable pathogens can be obtained from the nasopharynx. Despite high specificity, its sensitivity is relatively low, which can be attributed to various factors including prior antimicrobial treatment, the stage of the disease, the immune status of the patient, specimen collection and transport, and the choice of culture media [4]. With the introduction of PCR, the routine use of culture has decreased, and this is unfortunate since the bacterial isolates are still required to monitor the pathogen's evolutionary trajectory, its antimicrobial resistance profile, and possible alterations in virulence determinants associated with acellular pertussis vaccines.

2.2.2. PCR

PCR-based pertussis diagnosis is generally based on the insertion sequence (IS) element IS*481* that has been present in multiple copies within the genome of *B. pertussis*, and has been assumed to be specific to this pathogen. However, it should be noted that IS*481* can also be found in certain closely related species of *B. pertussis*, such as *B. holmesii* and *B. bronchiseptica*, which leads to variation in PCR specificity [15]. The use of dual targets has been helpful in overcoming non-specific cross--reactions within the *Bordetella* genus. For *B. pertussis*, IS*481* and the pertussis toxin promoter (*ptxP*) have been commonly employed, but it is important to acknowledge that the utilization of these genetic elements is not without limitations, as the *ptxP* region is prone to sequence variations [16]. Therefore, it is possible to conduct multitarget PCR assays to accurately differentiate *B. pertussis* from other members of the genus (Table 2). Moreover, it is essential to consider the interpretation of PCR results in conjunction with culture confirmation and clinical manifestations for a more accurate diagnosis.

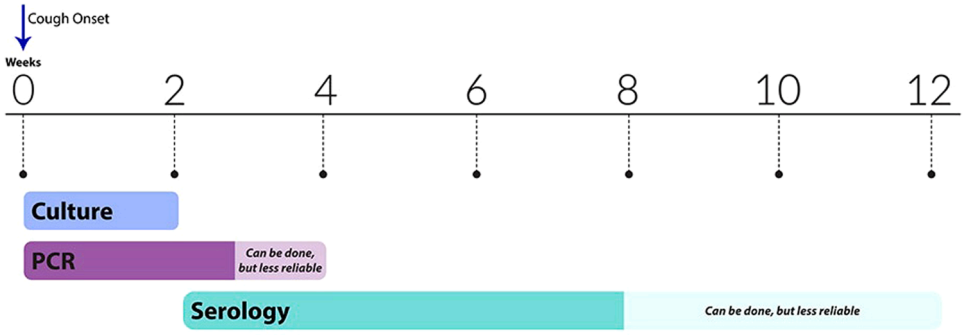

Fig. 1. Optimal timing of diagnostic tests for pertussis (cdc.gov/pertussis).

Table 2
Possible targets for detection of *Bordetella* DNA by PCR [17].

Target	Organism	Copy number per genome
IS481	*B. pertussis*	50–200
	B. holmesii	8–10
	Some *B. bronchiseptica*	<5
IS1001	*B. parapertussis*	~20
	Some *B. bronchiseptica*	1–7
IS1002	*B. pertussis*	4–9
	B. parapertussis	9
	B. bronchiseptica	1
ptxP	*B. pertussis*	1

2.2.3. *Serodiagnosis of pertussis*

Serologic assays have the advantage of being applicable at a later stage compared to culture and PCR, and the optimal timing for sampling is from 2 to 8 weeks, as this is when antibody titers reach their highest levels [14]. Following the successful isolation of *Bordetella pertussis* by Bordet and Gengou, a subsequent investigation was conducted to employ serological methods for the detection of agglutinating antibodies present in human sera [17]. Furthermore, apart from agglutination, various serological methods, including immunodiffusion, indirect immunofluorescence, indirect hemagglutination, and complement fixation, were employed to assess the immune response to *B. pertussis*. However, they have failed to demonstrate a satisfactory level of specificity and sensitivity [4]. Since the 1980s, traditional serologic assays have been replaced by modern techniques, such as enzyme-linked immunosorbent assay (ELISA) or multiplex immunoassays. In these methods, the detection of pertussis infection has commonly relied on the utilization of specific antibodies targeting *B. pertussis* toxins, such as pertussis toxin, pertactin, and filamentous hemagglutinin, in serum or oral fluid samples. The measurement of pertussis toxin antibodies is highly recommended in routine diagnosis since WHO provides a laboratory handbook for pertussis diagnosis with ELISA [18].

3. Revolutionizing healthcare: the power of microfluidic point-of-care testing

In the dynamic landscape of healthcare, the integration of cutting-edge technologies has facilitated the emergence of revolutionary

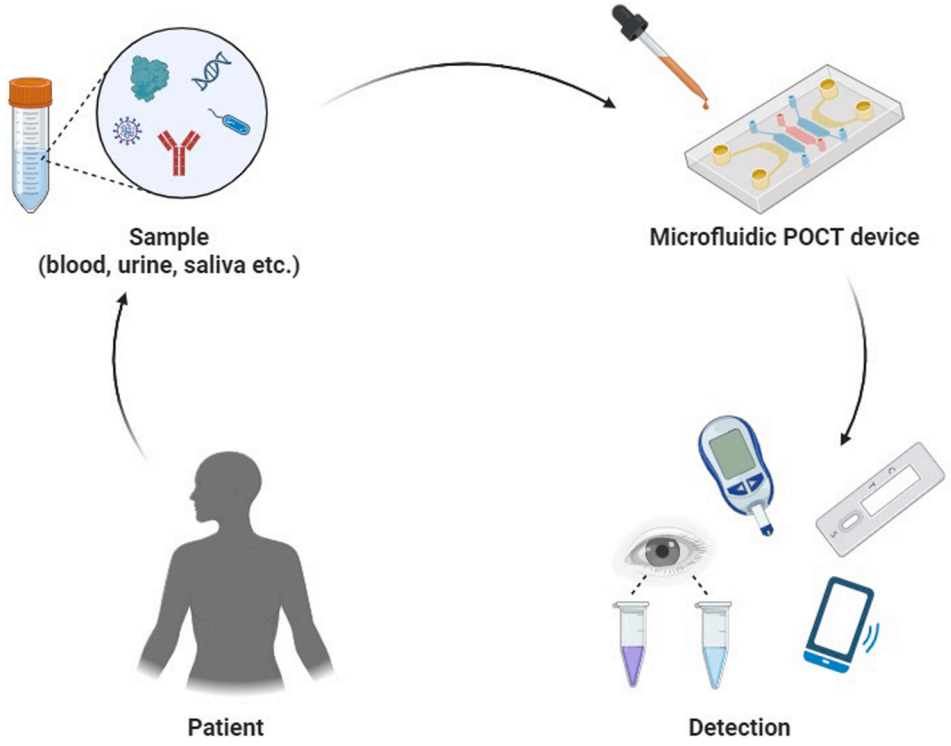

Fig. 2. A schematic representation for diagnosis of diseases using microfluidic POCT. (Figure created with BioRender.com).

diagnostic methodologies. One of the notable advancements in the field is the introduction of the microfluidic POCT approach, which has emerged as a game-changer, offering rapid and efficient solutions to the diagnosis of diseases (Fig. 2). The recent worldwide spread of COVID-19 has emphasized the importance of this kind of approach for timely prevention and control of diseases, especially during a pandemic.

The field of microfluidic POCT centers on the manipulation of small amounts of fluids on a microchip, enabling the incorporation of several laboratory operations into a compact and portable device. Microfluidic systems possess the ability to enhance speed and efficiency while concurrently minimizing the consumption of reagents or samples. It is possible to fully automate the process, resulting in reducing human interference and mitigating the risk of contamination. In microfluidic POCT diagnostics, two prevalent technologies are frequently employed, namely the Lateral Flow Assay (LFA) and Nucleic Acid Amplification. In LFA tests, DNA/RNA oligonucleotides or antibodies that are tagged with a chromophoric molecule can be used as biorecognition elements, which form a mobile conjugate when encountering the target. A range of biological fluids such as blood, serum, saliva, tears, and urine can serve as viable samples for the diagnosis and prognosis of diseases like cancer, as well as detecting the levels of certain molecules such as glucose and beta human chorionic gonadotropin (pregnancy hormone) [19]. Although LFA tests offer several benefits, including portability, cost-effectiveness, and extended shelf life, their accuracy can be compromised, particularly due to the quality of biorecognition elements that is affected during preparation [6]. While LFA tests have been extensively employed, their sensitivity and specificity have been limited beyond a certain threshold. The integration of microfluidic technology with nucleic acid diagnosis stands out as a highly promising application as it simplifies the intricate processes associated with amplification and detection, effectively eliminating the need for bulky instrumentation. In nucleic acid amplification-based tests, the DNA/RNA sequences specific to the target are used to generate multiple copies, which are subsequently detected by a probe to produce a signal [20]. For amplification, PCR or loop-mediated isothermal amplification (LAMP) can be employed. The latter method is particularly advantageous since the amplification process only needs to be completed at a constant temperature, unlike PCR, which necessitates varying temperature settings [21]. Despite the use of a very small amount of sample in these assays, they are confronted with several challenges, such as the need for heating and cooling systems in PCR-based microfluidic POCT, sample-dependent amplification performance, the presence of amplification inhibitors in unprocessed samples, and the need for more than three primers in LAMP-based tests [6,22,23].

The field of microfluidic POCT has seen major advancements, such as the development of glucometers and pregnancy tests, which showcase its potential. However, it is important to note that accomplishing all stages, from sample collection to data interpretation, remains a considerable challenge for the detection of infectious diseases [24]. One prominent issue is the requirement for ultrahigh specificity and sensitivity in the diagnosis of infections, in addition to error-free interpretation, good shelf life, and easy storage of reagents. Although numerous enterprises have exerted considerable effort in the translation of academic research findings into practical applications, further exploration is anticipated in order to effectively convert microfluidic POCT-based technologies into clinically valuable products.

3.1. Advancing pertussis detection: microfluidic POCT approaches

Initial investigations on the use of microfluidic approaches for pertussis diagnosis have primarily centered around the incorporation of PCR into microfluidic technology. In 2006, Easley et al. (2006) described a glass-based microfluidic system that could operate with whole blood as a crude sample [25]. The system consisted of solid-phase extraction (SPE), target sequence amplification by PCR, and subsequent electrophoretic separation and detection steps, all of which were completed in less than 30 minutes. The system was subjected to testing using whole blood samples of infected mice to identify *Bacillus anthracis* and a nasal aspirate sample of a patient to detect *B. pertussis*. The results indicated the presence of *B. anthracis* in 750 nl of blood and the presence of *B. pertussis* in 1 μl of nasal aspirate. In this system, the reagents utilized for DNA extraction in the SPE domain should have been isolated, as these chemicals could inhibit PCR. Although microfluidic-based methods provide promising outputs, certain critical processes, such as sample preparation prior to amplification, remain limiting factors. In order to address this particular obstacle pertaining to *B. pertussis*, de la Rosa et al. developed a microfluidic device that combined a glass microchip with circular aluminum microelectrodes, a tailored printed circuit board, and a microfluidic delivery and collection system for samples [26]. Positive dielectrophoresis was employed in conjunction with pulsed voltage to achieve the electrodisruption of bacterial cells. The device effectively lysed *B. pertussis* cells without causing any damage to genomic DNA, hence making its integration into microfluidic POC devices possible for chip-based sample preparation. For the detection of PCR-amplified genes, capillary electrophoresis (CE) has emerged as a promising tool that can be used with microfluidic technology. Prakash et al. (2008) designed a microdevice that consisted of nine PCR sample chambers equipped with inlet and outlet ports, which were connected to the CE section via fluidic tunnels [27]. It was based on integrated PCR-CE chip technology, and the researchers were able to successfully detect the presence of *B. pertussis* DNA when it was isolated from only 2 CFU/ml. The drawback of the study was to use purified *B. pertussis* DNA for the determination of the detection limit of the chip. Further studies could be conducted to integrate an on-chip sample preparation process into the device.

In recent years, studies have focused on improving the PCR-related parts of microfluidic devices for the detection of *B. pertussis*. In a study conducted by Hilton et al. a bead-based PCR was developed and integrated into a microfluidic device that consisted of a glass slide equipped with a resistive heater and a temperature controller [28]. Within this particular system, one set of the primers was attached to a streptavidin-coated microbead through dual-biotin linkage, and the other primer set was labeled with carboxyfluorescein. Upon the interaction between template DNA and the primer-coated microbeads, PCR amplification was initiated, and a fluorescent signal was detected at the end of the process. This system could detect a 181-base pair part of the *B. pertussis* genome at a concentration of 1 pM in 10 cycles of PCR within 13 minutes. One notable advantage of the bead-based PCR microfluidic device is that the amplified single-strand DNA can be obtained from the beads and used for further analysis. Another advancement in the DNA amplification part of microfluidic devices is the use of LAMP, in which DNA amplification is performed using four or six different primer sets at a constant temperature [29]. Since its introduction in 2000, this method has been adapted to the detection of *B. pertussis*, which paved the way for the studies of microfluidic devices integrated with LAMP [30]. In 2019, the first study was conducted by integrating LAMP into a low-cost microfluidic device for *B. pertussis* detection [5]. In this system, the device was assembled through a hybrid approach that consisted of both a paper substrate and a transparent polydimethylsiloxane substrate, ensuring better primer distribution and more stable diagnostic performance. Also, an internal battery-powered heater was devised, effectively eliminating the need for external power for the operation and enabling rapid POCT detection of pertussis. Additionally, it had a limit of detection as low as five DNA copies per LAMP zone, and the results could be evaluated by the naked eye under a portable UV light pen within one hour. The device allowed direct detection of the pathogen from nasopharyngeal samples that were prepared through mixing with a lysis buffer and incubation at room temperature for ten minutes, providing a centrifuge-free lysis procedure. The researchers also validated the LAMP-based microfluidic POCT device through the investigation of 100 clinical nasopharyngeal swabs and aspirates, which were previously confirmed to be either negative or positive for *B. pertussis* by the

real-time PCR method [31].

Various commercial tools are available for the detection of a wide range of pathogens, such as bacteria and viruses, utilizing microchip technology [7]. Luminex Verigene® and Biofire FilmArray® are two examples of microfluidic-based molecular diagnostic systems that have demonstrated efficacy in rapidly identifying *B. pertussis* in various sample types. Although they serve as rapid diagnostic tools, they lack portability, rely heavily on consumables, and are not readily affordable.

4. Conclusion

Pertussis, as a vaccine-preventable disease, is subject to mandatory reporting in the majority of countries worldwide. The use of PCR is a routine procedure for pertussis diagnosis in many industrialized nations. WHO and relevant organizations express valid concerns with inadequate or absent surveillance of pertussis, particularly in countries where it is of utmost importance. The main reason behind the lack of proper surveillance is insufficient laboratory infrastructure in these nations. Therefore, low-cost and portable POC assays for the detection of *Bordetella pertussis* are a valuable approach to generating data concerning the prevalence of pertussis in regions with the highest disease burden.

The current stage of microfluidics technology is characterized by several challenges, including optimization of reactions, complex channel design, valve failure, and difficulty in recyclability, which serve as a significant barrier to widespread implementation. Thus, further improvements are required to establish user-friendly, less error-prone, reproducible, and affordable microfluidic platforms. Despite these challenges, microfluidic-based diagnosis is increasingly recognized as a valuable tool in the management of infectious diseases, both presently and in the future.

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Çiğdem Yılmaz Çolak: Writing – review & editing, Writing – original draft, Supervision, Investigation, Conceptualization.

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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