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Review

Differential Markers of Bacterial and Viral Infections in Children for Point-of-Care Testing

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Children suffering from infectious diseases, both bacterial and viral, are often treated with empirical antibiotics. Keeping in mind both the menace of microorganisms and antibiotic toxicity, it is imperative to develop point-of-care testing (POCT) to discriminate bacterial from viral infections, and to define indications for antibiotic treatment. This article reviews potential protein biomarkers and host-derived gene expression signatures for differentiating between bacterial and viral infections in children, and focuses on emerging multiplex POCT devices for the simultaneous detection of sets of protein biomarkers or streamlined gene expression signatures that may provide rapid and cost-effective pathogen-discriminating tools.

Assessing Infectious Disease among Children: The Need for Improved Diagnostic Tests

Bacterial and viral infections are the most common reasons that children receive medical care in clinics or small hospitals [1,2]. Because the symptoms exhibited by patients with bacterial and viral infections are similar, symptom-based examinations and history-taking are usually insufficient to distinguish between bacterial and viral infections, even by experienced doctors [3]. However, it is essential to make this distinction, and to make it early, because the pediatric population has a characteristically weak immune system and is prone to rapid disease spread. Although the treatment choices are substantially different for bacterial and viral infections, children are often treated with **empirical antibiotics** (see Glossary) owing to the lack of rapid and accurate testing. Despite the urgency of early intervention, current diagnostic methods (Box 1) are either inaccurate or time-intensive. This may lead to delayed diagnosis and antibiotic misuse, which increase morbidity rates as a result of antibiotic-associated adverse events, **antibiotic resistance**, and other potentially confounding problems [4,5]. Common acute adverse events caused by antibiotics include allergic reactions (even life-threatening anaphylaxis and Stevens–Johnson syndrome), neurological complications, and psychiatric disturbances [6]. In addition, the frequent use of antibiotics in early childhood may increase the occurrence of unfavorable long-term consequences such as diabetes and obesity [7–9]. Moreover, antibiotic resistance can negatively affect both individual patient health and public health (and is considered to be one of the biggest global public health challenges) [10]. Finally, prolonged hospital stays and extended medical treatment for antibiotic-resistant infections may increase the financial burden [11–13].

Although current **biomarker**-based biochemical analyses based on **procalcitonin** (PCT) and **C-reactive protein** (CRP) can provide some clues for differential diagnosis between bacterial and viral infections, they cannot be used for treatment guidance because of their relatively low sensitivity and specificity [14,15]. Moreover, patients with underlying diseases such as cystic fibrosis, asthma, and bronchiectasis tend to have bacteria (e.g., *Staphylococcus aureus* and *Pseudomonas aeruginosa*) contained in biofilms that form in respiratory tract tissues, and viral infection could even facilitate biofilm formation. Bacteria–virus interactions and their relationships to

Highlights

Bacteria and viruses activate or inhibit different signaling pathways in the cells they infect, and further give rise to different host transcriptional signatures as well as to unique protein biomarkers.

Many of the newly evaluated protein biomarkers, especially in combination, have better discriminative value for distinguishing between bacterial and viral infections than the biomarkers that are currently used for examining infections in children.

The transcriptomes of children undergo remarkable changes when they are infected by different types of bacteria and viruses. Approaches based on host-derived DNA/RNA signatures can accurately discriminate bacterial from viral infections.

Emerging multiplex POCT techniques allow simultaneous testing of protein- or gene-based biomarkers in an outpatient setting.

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Box 1. Widely Used Current Diagnostic Methods for Infectious Diseases

Culturing

Culturing is defined as the growth of microorganisms in culture medium under controlled conditions. It has long been recognized as the diagnostic gold standard for diagnosing bacterial and fungal infections, even though it normally takes several days to complete. However, the emerging MALDI-TOF MS technology (matrix-assisted laser desorption ionization time-of-flight mass spectrometry) has largely revolutionized the workflow for culture methods, and this method has been gradually improved to provide timely infectious source information [1].

PCR

PCR is a technique for amplifying a specific DNA to create measurable amounts. This method can provide highly sensitive diagnostic information for microbial identification within 4–8 h. However, it is costly and requires sophisticated instrumentation.

Microscopy

Microscopy, usually in combination with staining techniques, can be used to provide early observation of infectious pathogens. However, diagnostic accuracy is easily influenced by specimen quality and technician experience.

Radiology

Radiological imaging (e.g., X rays) is widely used to assist in diagnosing the presence and severity of respiratory infections. However, radiation exposure is a concern for pediatric population examinations.

Biomarker Testing

Testing for specific molecular alterations provides a rapid and cost-effective method for the diagnosis of infectious disease. However, doctors usually cannot make a diagnosis from single biomarker data because of the relatively low specificity.

biofilm formation make causative pathogen diagnosis more challenging [16]. These factors heighten the need for distinct biomarkers and the development of novel **point-of-care testing** (POCT) to discriminate between bacterial and viral infections in pediatric patients. Such an approach would facilitate more cautious selection of appropriate therapy [17,18].

From sample collection to biomarker detection, there are substantial differences between the methods and expectations for diagnosing infectious diseases in pediatric (age ≤18 years) and adult populations. Regarding respiratory tract infections, for example, specimen collection, sputum microscopy, and culturing are commonly used for diagnosing the causes of pneumonia in adults, but these methods are rarely used in children owing to difficulties in obtaining proper specimens [19]. For pediatric patients, bronchoalveolar lavage fluid and blood specimens (sterile specimens) would be more suitable for biochemical analysis to detect pneumonia because these methods avoid contamination by upper respiratory tract flora [20]. Furthermore, the health characteristics of children, including (i) relatively immature immune systems that are especially vulnerable to bacterial and viral infections as well treatment side-effects, (ii) the different epidemiology and etiology of infections compared with adult populations [21], and (iii) the presence of different host-derived protein biomarkers and **gene expression signatures** in infected children compared with infected adults [22–25], make them a special population when facing infectious diseases.

Over the past few years a growing number of host-derived biomarkers demonstrating superior ability to diagnose infectious etiology among children have been developed. These biomarkers have included bacteria/virus-induced host proteins or gene expression signatures (Figure 1) [26,27]. Employing a combination of a set of protein biomarkers and/or gene expression signatures has further been shown to improve diagnostic accuracy [15]. Possible target resources for identifying the cause of infection include host- and pathogen-derived biomarkers such as gene expression signatures, proteins, proteases, metabolites, etc. [28,29]. However, some of these biomarkers, including most pathogen-derived biomarkers, can identify specific infectious sources but do not generally differentiate between bacterial and viral infections, which is

Glossary

Antibiotic resistance: bacterial resistance to an antibiotic to which they were previously susceptible. Treatment of resistant bacteria is usually difficult and may require higher doses and more toxic antibiotics, and in some cases no effective antibiotics can be applied.
Biomarker: a biologically derived marker that can provide information about the organism. Biomarkers are often evaluated to assist disease diagnosis, pathogen identification, and treatment responses.
C-reactive protein (CRP): a plasma protein produced by the liver whose levels rises during inflammation.
Empirical antibiotics: antibiotics prescribed to patients based on a doctor's experience and in the absence of confirmed diagnosis. Application occurs before identification of disease etiology.
Gene expression signatures: a single gene or a series of genes that display a characteristic pattern of expression that appears in specific physiological or pathological conditions.
Immunoassay: a biochemical test that detects or quantifies target molecules in a sample through the specific reaction between an antibody and an antigen.
Lateral flow assay (LFA): an immunoassay adapted for use in a paper strip format to measure the presence or concentration of target molecules. The liquid sample usually moves through the paper strip via capillary action, and results are usually presented at a control line (to confirm that the test has worked correctly) as well as at one or more test lines (indicating that target analytes are present in the sample).
Microarray: a multiplex lab-on-a-chip for the simultaneous analysis of large numbers of biomarkers. Biological molecules (e.g., DNA and protein) are usually present as a 2D array and are used as probes to detect specific sample targets.
Microfluidics: a technology that involves accurate control of fluid movement at the microliter scale.
Multiplex POCT: the simultaneous detection of multiple biomarkers in a sample.
Point-of-care testing (POCT): medical diagnostic testing delivered to patients at the time and place of care. POCT is usually performed outside
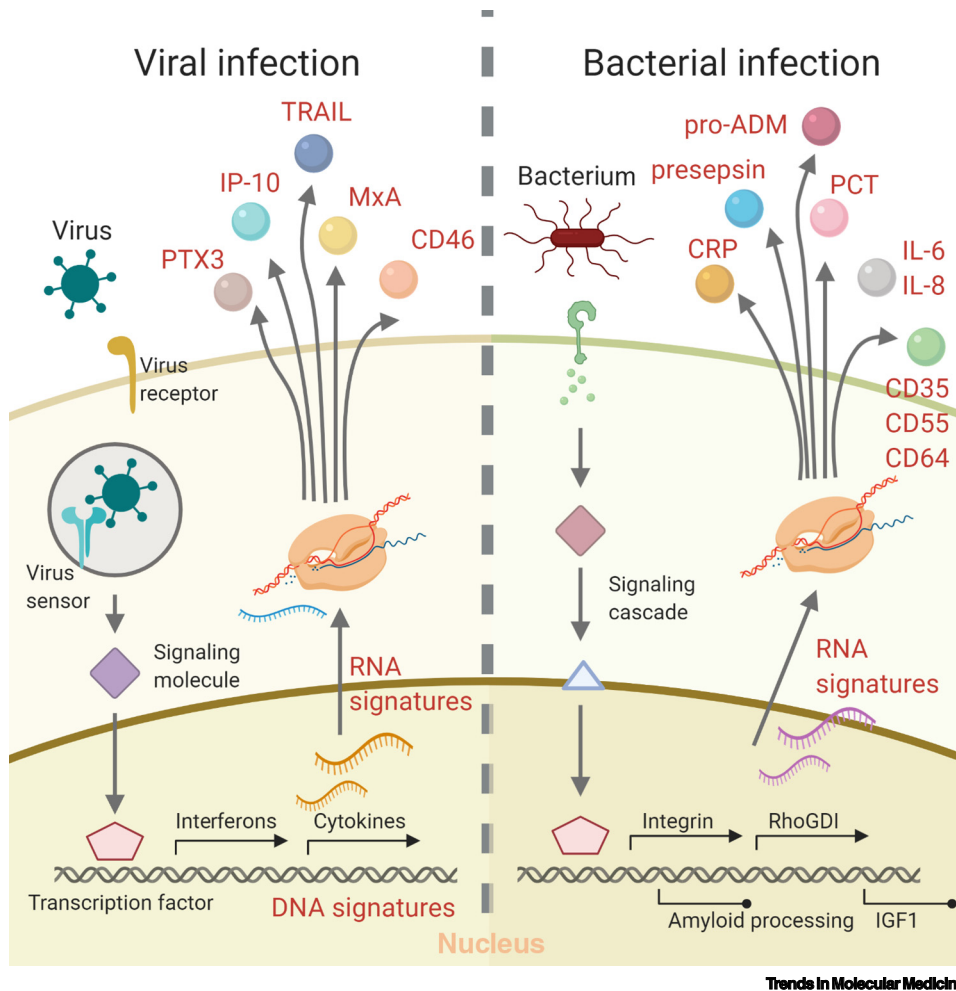
Figure 1. An Overview of Cellular Responses to Bacterial and Viral Infections. Bacterial and viral infections can induce different recognition and signaling pathways. Regarding gene expression signatures, viruses are more likely to trigger interferon (IFN)-related signatures, whereas bacteria are more likely to induce integrin-related signatures. TRAIL, IP-10, PTX3, MxA, and CD46 are thought to be viral infection markers, whereas CRP, PCT, presepsin, pro-ADM, IL-6, IL-8, CD35, CD55, and CD64 are thought to be bacterial infection markers. All of these host-derived RNA and protein biomarkers could be used to differentiate between bacterial and viral infections. Figure created with BioRender.com.

laboratory-based settings to improve accessibility for the patients.
Procalcitonin (PCT): a peptide precursor of calcitonin whose levels usually rise in response to inflammation caused by bacterial infections.

particularly important for antibiotic treatment decisions [30,31]. Therefore, we focus on host-derived protein-based biomarkers and gene expression signatures because they have recently been shown to be promising biomarkers for distinguishing between bacterial and viral infections. In addition, although some recently studied biomarkers have displayed outstanding discriminatory power regarding bacterial and viral infections, the clinical performance of these biomarkers has not been fully investigated. We focus here on developments in POCT for rapid discriminative tools. Although rapid discriminative tools are favored across all patient cohorts, POCT is especially important for pediatric populations because it can be used in acute outpatient settings, the primary battlefield for pediatric infections. Pediatric populations are also susceptible to fungal and parasite infections under immunocompromised conditions [32]. However, because these infections are relatively rare, we focus here on differentiation between bacterial and viral infections. We first review protein-based biomarkers and gene expression signatures, and discuss

experimental requirements. We then review currently available **multiplex POCT** to distinguish between bacterial and viral infections in children, and we discuss future developments in determining infectious etiologies.

Bacterial Protein Biomarkers

Because host proteins are readily amenable to rapid and quantitative measurements using well-established technologies, they are commonly evaluated biomarkers in routine pediatric care [14,33] (Table 1).

Established Biomarkers

CRP and PCT are the most frequently evaluated and indicative biomarkers for identifying bacterial infections in children because their levels are higher in bacterial infections than in viral infections [15,33–36]. Although CRP and PCT can be used with great accuracy to detect many pediatric infections such as pneumonia and meningitis [37–43], some studies have shown inconsistent results [44], and this undermines their reliability as a sole predictor. The large range of CRP values is especially a weakness with regard to differential diagnosis [14].

In addition to CRP and PCT, interleukin-6 (IL-6) and interleukin-8 (IL-8) are frequently evaluated biomarkers for diagnosing bacterial infections in children because of their elevated levels during infection [18,33,45]. However, it is difficult to say that IL-6 and IL-8 are more sensitive or specific than CRP or PCT because both poorer and better results have been obtained. Recent studies have shown that IL-6 tests are more sensitive and specific for identifying sepsis in children, but in regards to differential diagnosis of bacterial and viral infections in children with respiratory tract infections or fever without a source, CRP tests are more accurate [25,46–48]. Moreover, it has been reported that the level of serum IL-6 in children is higher in Gram-negative bacteremia than in Gram-positive bacteremia [46]. This may lead to inconsistent results when testing for IL-6 because Gram-negative and Gram-positive bacteria levels are associated with different infectious diseases. Further studies will be necessary to examine the performance of these biomarkers and their association with different types of infectious disease. Although CRP and PCT remain the primary markers for distinguishing between bacterial and viral infections, they are unlikely to fully aid physicians in ruling out all potential bacterial infections. Nevertheless, because of the similar biological pathways of these bacteria-induced proteins (i.e., PCT, CRP, IL-6, and IL-8), there is value in using a combination of biomarkers to provide superior sensitivity and specificity [15,49].

New Potential Markers

Biomarkers that have recently been found to have potential value as bacterial infection markers include IL-27, CD35, CD64, presepsin, and pro-adrenomedullin (proADM). IL-27 is an immunoregulatory cytokine that induces the differentiation and modification of T cells. A high level of IL-27 was found to predict bacterial infection in critically ill pediatric patients [50]. CD35 is a membrane-bound complement regulator, and CD64 is an integral membrane glycoproteins of white blood cells. Both CD35 and CD64 are present at higher levels in children with bacterial versus viral lower respiratory tract infections [51]. In addition, presepsin, an immunologic biomarker related to bacterial phagocytosis, specifically elevates in response to bacterial infections [52]. For this reason, presepsin has recently been reported to be useful for predicting infection with high diagnostic accuracy in children with sepsis and bacterial ventriculitis [53,54]. Adrenomedullin, a peptide secreted by vascular endothelial cells and musculature, is primarily involved in the process of immune modulation and vasodilation. Testing for its precursor, proADM, shows high sensitivity and specificity for differentiating bacterial community-acquired pneumonia (CAP) from non-bacterial CAP that could be virus-, fungus-, or parasite-induced pneumonia [55]. Although promising, most studies focused only on distinguishing between bacterial and non-bacterial infections because this information is

Table 1. Protein Markers as Clinical Markers of Pediatric Infections[a]

Biomarker	Clinical syndrome	Sample type	Protein detection method	Study size	Bacterial vs. non-bacterial	Viral vs. non-viral	Bacterial vs. viral	Sensitivity (%)	Specificity (%)	Cut-off	Refs
Traditional markers											
C-reactive protein	PB-CAP	Serum		110			+	64.4	69.4	≥7.4 mg/l	[67]
	Meningitis	Serum	Immunoturbidimetric assay	370	+		+	91.16	100	57 mg/l	[104]
	DCLD	Serum	Immunoturbidimetric assay	164	+			86.8	73.8	>0.6 mg/dl	[36]
	Pneumonia Bacterial sepsis CAP	Serum Serum Serum	Immunoturbidiometric and immunofluorescence assays	230–842 80 88	1/3 + +		3/3	75–90 80 100	68.1–82 65.7 70.5	37.1–80 mg/l >13.49 mg/l >100 mg/l	[37–39] [47] [55]
Procalcitonin	Meningitis	Serum	ELISA	370	+		+	66.7	59.3	60 ng/dl	[104]
	SBI and IBI	Serum	ELISA	2047	+			90	78	≥0.3 ng/ml	[40]
	DCLD	Serum	Immunoluminometric assay	164	+			86.8	91.7	>0.5 ng/ml	[36]
	CKD	Serum	ELISA	102	+			94.1	87.9	0.5 ng/ml	[35]
	Meningitis	Serum	Immunoluminometric assay	616			+	96	89		[42]
	Bacteremia	Serum	Electrochemiluminescence immunoassay	65	+			81	68	0.25 µg/l	[46]
IL-6	Bacterial sepsis	Serum	ELISA	80	+			100	62.86	>51.29 pg/ml	[47]
	Bacteremia	Serum	Electrochemiluminescence immunoassay	62	+			64	88	196.6 ng/l	[46]
New potential markers											
IL-27	Bacterial infection in critically ill patients	Serum		702	+			12	95	5 ng/ml	[50]

MxA	RSV and rotavirus	Whole blood	ELISA	193	-	+	+	96.4	66.7	>200 ng/ml	[58]
	RTI	Whole blood	EIA	153		+		92	77	175 μg/l	[59]
Lactate	Meningitis	CSF	Standard enzymatic test	370	+		+	96.2	78.4	30.2 mg/dl	[104]
Presepsin	LOS	Whole blood	Chemiluminescent enzyme immunoassay	40	+			94	100	885 ng/l	[105]
	CRBSI	Serum	ELISA	138	+			100	93.8	990 pg/ml	[106]
	Suspected bacterial meningitis or ventriculitis	CSF	Chemiluminescent enzyme immunoassay	18	+			84.2	82.1	625 pg/ml	[54]
Pro-adrenomedullin	CAP	Serum	TRACE	88	+			100	70	>0.16 nmol/l	[55]
	Sepsis	Plasma	ELISA	60	+			93.3	86.7	4.3 nmol/l	[65]
Combined evaluation											
TRAIL, IP-10, and CRP	RTIs and fever without source	Serum	Immunoassay	493			+	93.5	94.3	<35 viral >65 bacterial	[25]
	LRTIs or fever without source	Serum	ELISA for TRAIL and IP-10; immunoassay for CRP	443		+		86.7	91.1		[4]
Procalcitonin + IL-6	SBI	Serum	Electrochemiluminescence immunoassay	126	+			93.84	96.72		[49]

[a]Abbreviations: CAP, community-acquired pneumonia; CKD, chronic kidney disease; CRBSI, catheter-related bloodstream infections; CRP, C-reactive protein; CSF, cerebrospinal fluid; DCLD, decompensated chronic liver disease; EIA, enzyme immunoassay; ELISA, enzyme-linked immunosorbent assay; GBS, group B Streptococcus; IBI, invasive bacterial infection; IL, interleukin; IP-10, interferon γ-induced protein 10; LOS, late-onset sepsis; LRTIs, lower respiratory tract infections; MxA, myxovirus resistance protein A; PB-CAP, probable bacterial infection community-acquired pneumonia; RSV, respiratory syncytial virus; RTI, respiratory tract infection; SBI, severe bacterial infection; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; TRACE, time-resolved amplified cryptate emission technology.

sufficient to assist clinicians in making antibiotic-based treatment plans. However, additional research will be necessary to compare differences among coinfection cases.

Protein Biomarkers of Viral Infection

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), an apoptosis inducer, and interferon (IFN)-γ-induced protein-10 (IP-10), a chemokine involved in inflammation and angiogenesis, have been shown to be activated in response to a variety of viral infections and are significantly lower in bacterial infections [15,25,33,56,57]. In addition to TRAIL and IP-10, myxovirus resistance protein A (MxA), that is responsible for inhibition of virus replication in cells, has emerged as biomarker of viral infections. MxA levels are significantly higher in patients with viral (respiratory syncytial virus and rotavirus) versus bacterial infections and uninfected controls [58]. Higher levels of MxA in children with symptomatic respiratory virus infections (including rhinovirus infections) indicate that it could be a valuable marker for detecting viral respiratory infections in children [59]. However, it remains unclear whether MxA can be used to distinguish between bacterial and viral infections. Pentraxin 3 (PTX3), an acute-phase protein that takes part in complement activation and pathogen recognition, has been proposed as a potential indicator of viral respiratory tract infection in children [60]. Moreover, it can reflect disease severity, including peak fever temperature and fever duration before admission [61]. Another recently reported viral biomarker, CD46, is an inhibitory complement receptor. An increase in the expression of CD46 in monocytes may be used to indicate the presence of viral infections in febrile children [62].

Biomarker Combinations

The combined triple host protein assay comprising TRAIL, IP-10, and CRP for distinguishing between bacterial and viral infections is superior to single-biomarker methods [4,15,25,63], and has 93.5% sensitivity and 94.3% specificity [25]. Another approach using combinations of CRP + CD35 and CRP + CD64 was found to provide better discriminative power than a single biomarker or CRP + PCT and CRP + IL-6 for the differentiation of bacterial and viral lower respiratory tract infections in children [51]. Although some studies found that combined assessment did not improve assay results [64], combined approaches offer several promising possible avenues for improved diagnostic differentiation.

Although several studies have evaluated new markers or their combinations, most only address a specific infectious disease or specimen type, and thus do not allow generalization. In addition, patients with mild/localized infections or severe infections complicated by systemic involvement may display different levels of such biomarkers in serum [55,65]. Last but not least, underlying diseases (e.g., immunodeficiency or cancer) may impact on the performance of biomarkers because their levels generally rise in response to different inflammatory processes [46,66]. Therefore, further studies examining a variety of infectious conditions and coinfections are warranted.

Combined evaluation methods can improve diagnostic accuracy in identifying a single infection by combining markers with similar properties, or by combining complementary markers. The discriminatory power of CRP testing is relatively low when applied in pneumonia cases [37–39,55,67]. Notably, CRP is also elevated in viral upper respiratory tract infections within the first 4 days of illness, indicating that the host response is more complex in the case of pneumonia [36]. Thus, we propose to examine protein-based biomarkers in a variety of infectious diseases, especially pneumonia, before utilizing them for diagnostics across a broad spectrum of diseases.

Gene Expression Signatures

Although host protein biomarkers and PCR analysis of microbial pathogens are commonly used in clinical practice, it is difficult to make antibiotic decisions based on these analyses because of

their relatively low sensitivity and specificity. Zhu *et al.* first reported host gene expression changes in response to human cytomegalovirus infection [68]. Following this discovery, many studies demonstrated that the host genetic transcriptome undergoes remarkable changes during infection by different types of bacteria and viruses [69]. Recently, researchers have made efforts to control epidemic infections based on host transcriptional signatures. Kanniappan *et al.* first used small RNAs (sRNAs) to detect *Mycobacterium tuberculosis*, demonstrating sensitivity and specificity of over 95% [70]. In addition, other infectious diseases such as enteric fever and Kawasaki Disease can also be distinguished via specific gene expression signatures [71,72]. The use of these genetic biomarkers will change the current clinical diagnostic criteria for many infectious diseases.

Potential Host Transcriptional Biomarkers

Analysis of host transcriptional signatures is a promising strategy to discriminate between bacterial and viral infections in children and curb the overuse of antibiotics (Table 2). When children encounter infections they display different transcriptional signatures compared with adults [22–24]. Thus, there is a need to develop specific diagnostic tests tailored to children. Hu *et al.* identified 260 and 1321 host transcriptional signatures that were significantly up- or down-regulated by viruses and bacteria, respectively, in febrile children. They also found that the IFN signaling pathway was particularly activated by viral infection, whereas the integrin signaling pathway was uniquely triggered by bacterial infection [73]. This led to a major shift from pathogen-centric genetic detection towards host transcriptional signatures for identifying the etiology of infectious disease. A total of 420 different host transcriptional signatures were discovered. In addition to genes underlying innate and adaptive immunity, diverse genes that mediate cell growth, metabolism, and signal transmission been found to respond to invasive bacteria and viruses [74,75] (information regarding host genes is summarized in Table S1 in the supplemental information online). Among these host transcriptional signatures, IFI44L is the best-studied host transcriptional signature because it demonstrated significant upregulation in viral infection in 1058 infectious children [26,76–79]. IFI44L is an IFN-stimulated gene (ISG) that inhibits the replication of diverse viruses [80]. The combined discriminative value of using IFI44L and FAM89A to distinguish between bacterial and viral infections has been studied in febrile infants and children, and in children with diarrhea. Results exhibited 68–100% sensitivity and 84–96.4% specificity. A relatively low sensitivity (68%) was found when these markers were used to differentiate between viral and bacterial diarrhea using the Gene Expression Omnibus (GEO) database [77]. Further, two other studies using the GEO database found relatively low sensitivities for differentiating between bacterial and viral infections [17,22]. Although database analysis can save cost and time, error correction must be performed carefully to provide accurate results. It should also be noted that both infection type and severity may influence the performance of gene expression signatures. Further, although the immune system of infants is relatively immature at birth and requires months to generate an adequate host response [81], several studies have shown that host transcriptional signatures can be accurate biomarkers for discriminating between bacterial and viral infections in infants less than 60 days old [75,78,79].

Future Directions from Bench to Bedside

The detection of specific host transcriptional signatures in response to bacteria or viruses is an attractive alternative approach for the diagnosis of infectious diseases in pediatric patients because they can provide precise information. However, the detection of a large group of biosignatures does not meet the clinical need for a rapid and simple POCT. Therefore, current studies have aimed to streamline diagnostic host transcriptional signature assays, such as through a four-biomarker blood signature [19], a two-transcript host RNA signature [67], and a qPCR single-gene expression assay to differentiate between viral and bacterial infections

Table 2. Host Transcriptional Profiling Studies for Discriminating between Bacterial and Viral Infections in Pediatric Patients[a]

Patient characteristic	Sample type	mRNA detection method	Gene number	Patient number	Bacterial vs. non-bacterial	Viral vs. non-viral	Bacterial vs. viral	Other group of patients	Accuracy in validation	Refs
Acute respiratory illness	Whole blood	RT-PCR	41	151	+	+		Coinfection, neither	B vs. non-B: 88% V vs. N: 81%	[23]
Febrile children	Whole blood	RT-PCR	2 1	35			+	Healthy	Sensitivity/specificity B vs. V: 90.9%/85.7% (two genes) B vs. V: 90.9%/92.8% (one gene)	[76]
Acute diarrhea	Whole blood	GEO database	2	174			+		Sensitivity/specificity B vs. V: 68%/84%	[77]
Acute respiratory illness	Nasopharyngeal aspirates	RT-PCR	184	58	+	+	+	Coinfection, indeterminate	Bootstrap values >50%	[84]
Intensive care children (bacterial infection as sepsis)	Whole blood	GEO database	4	69		+	+	Coinfection, neither	AUC in train B vs. V: 0.76/V vs. N: 0.91	[22]
Febrile Infants <60 days	Whole blood	GEO database	2	200			+		Sensitivity/specificity B vs. V: 88.8%/93.7%	[78]
Acute respiratory illness	Whole blood	Microarray	103	215	+	+	+	Neither, healthy	87%	[107]
Febrile infants <60 days	Whole blood	Microarray	66	279	+			Healthy	Sensitivity/specificity B vs. non-B: 87%/89%	[79]
Acute infection	Whole blood	GEO database EBI ArrayExpress	7	945	+	+			Sensitivity/specificity B vs. non-B: 94%/59.8% V vs. non-V: 53%/90.6%	[17]
Febrile children	Whole blood/peripheral blood mononuclear cells	Microarray	38 2	370	+	+		Indeterminate	Sensitivity/specificity B vs. V: 100%/86% (38 genes) B vs. V: 100%/96.4% (2 genes)	[26]
Critically ill children (sepsis vs. infection-negative systemic inflammation)	Whole blood	NGS RT-PCR	4	64					AUC in train 0.99	[108]
Acute infective infant	Whole blood	Microarray	52	89	+				Sensitivity/specificity B vs. non-B: 100%/97%	[75]

[a]Key and abbreviations: AUC, area under the ROC curve; B, bacterial infection; EBI, European Bioinformatics Institute; GEO, Gene Expression Omnibus; N, non-bacteria and non-viral infection; neither, inflammation without evidence of bacterial or viral infection; NGS, next-generation sequencing; V, viral infection.

[22,26,76]. In addition to the development of PCR techniques and portable devices that can test genetic biomarkers in an easy and rapid way [82], it is now possible to detect host transcriptional signatures in small hospitals and in developing countries. Moreover, nanotechnology molecular computation strategies (that assemble a computer using nanoscale molecular building blocks such as DNA instead of traditional silicon) have been used to analyze complex gene expression signatures in a point-of-care setting. This method has been validated in a 12 patient study, and the results showed 100% accuracy for differentiating between bacterial and viral respiratory infections by employing molecular computation in a general Eppendorf tube [83]. These efforts have made great strides towards the development of a POCT for distinguishing between bacterial and viral infections. Although most studies have only detected gene expression signatures in blood samples, immune signatures from nasopharyngeal aspirates can also be used to detect infection [84]. Non-invasive sample analysis provides several advantages; it is painless, enables self-collection, and offers superior safety for both patient and doctor. However, the quality of non-invasive samples (e.g., urine and saliva) is usually limited by fluctuations in sample flow rate. This directly influences test sensitivity and specificity. If this problem can be overcome, analyzing biomarkers in non-invasive samples would be a valuable approach for diagnosing and subsequently treating infants and children, and would facilitate the development of easy-to-use POCT.

In summary, host transcriptional signatures are good biomarkers for clinically discriminating between bacterial and viral infections in pediatric patients. However, genetic profiling of different infection types, and examination of complicating factors including infection severity and coinfection status, are necessary before clinicians can make practical and effective antibiotic decisions based on host transcriptional signatures.

Potential Multiplex POCT Discriminative Devices

Managing pediatric infections remains problematic because of diagnostic shortcomings and because of the related overuse of antibiotics, especially in resource-limited countries. Early diagnostic POCT devices must be developed to differentially diagnose bacterial and viral infections to prevent the potentially unnecessary and ineffective prescription of antibiotics. Tests using a combination of protein biomarkers or gene expression signatures would yield more accurate results than single biomarker tests for differentiating between bacterial and viral infections. As more biomarker sets are discovered for this purpose, the development of multiplex diagnostics, especially multiplex POCT for biomarker detection, may significantly improve healthcare efforts.

Although multiplex POCT is a relative new idea, its primary concept remains the same as traditional single biomarker-targeted POCT. Protein-based biomarker detection relies on **immunoassay** techniques that employ antibodies as capture probes. Genetic-based biomarker detection requires an initial amplification process, followed by DNA probe conjugation with reporters (e.g., fluorescent) for hybridization. A variety of different designs can then be added to these concepts to yield multiplex POCT (Figure 2). We briefly discuss the use of recently developed multiplex POCT to examine infectious diseases, with a focus on three primary types: (i) **lateral flow assays** (LFAs), (ii) **microfluidics**, and (iii) **microarrays**.

Lateral Flow Assays

Multiplex LFAs for infectious disease detection use different capture probes to detect several protein biomarkers or gene expression signatures in different areas of the device (Figure 2C2). Such assays benefit from an increased likelihood of providing at least one positive signal. This type of device has been used to detect CRP and serum amyloid A-1 (SAA1) associated with bacterial infections, and also to identify Zika and Dengue in the case of mosquito-borne disease [85,86].
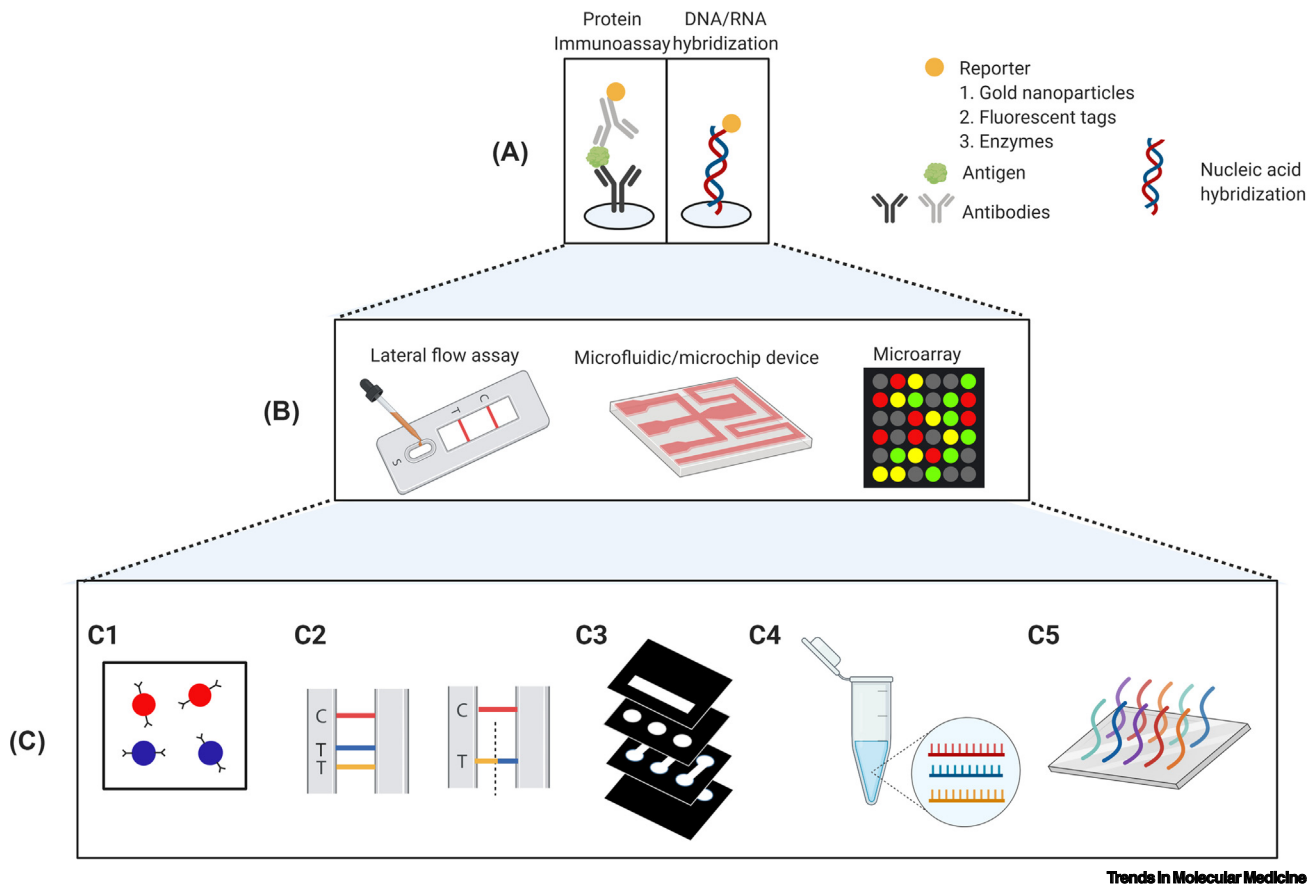
Figure 2. Scheme of Recent Multiplex Point-of-Care Testing for Infection Diseases. (A) Multiplex bacterial and viral infection biomarker detection is generally based on immunoassay (protein-based biomarkers) and hybridization (genetic-based biomarkers) techniques. (B) Several point-of-care methods such as lateral flow assays, microfluidic devices, and microarrays have been developed. (C) These methods have been expanded to multiplex assays based on the several innovations. (C1) Capture antibody conjugated with different reporters. (C2) Double test line or double flow path. (C3) Vertical flow design divides the sample into multiple wells. (C4) Isothermal nucleic acid amplification with multiple primers. (C5) Multiple immobilized probes. Figure created with BioRender.com.

Recently, a mature and commercially available multiplex LFA, FebriDx, was developed to differentiate between bacterial and viral acute respiratory infections with high sensitivity and specificity (~80–95%) [87–89]. This device is a disposable POCT that comprises two test lines coated with monoclonal anti-CRP and anti-MxA antibodies to simultaneously detect CRP and MxA. When using this device, a minimal invasive fingerstick blood sample is adequate to produce results within 10 minutes. Although FebriDx was thought to be a landmark in the development of multiplex POCT for infection discrimination, it is only indicated for use in patients with acute respiratory infections. Further studies verifying its utility for assaying different types of infectious diseases are needed.

Microfluidics

Multiplex microfluidics devices integrate multiple biomarker detection by controlling fluid flow to create separate detection regions, and many POCT methods for diagnosing infectious diseases apply this technology in different ways. Multiplex microfluidics for protein-based biomarkers are often integrated with immunoassay methodology. This approach has been used, for instance, to detect CRP and PCT to diagnose sepsis [90,91]. Another example is a paper-based microfluidics device that uses a unique 3D paper structure to detect multiple biomarkers

Clinician's Corner

Bacterial and viral infections are the most common diseases among pediatric populations and are often prescribed with empirical antibiotics in small outpatient settings such as clinics or small hospitals.

However, treatment strategies for bacterial and viral infections are completely different. Bacterial infections are usually treated with antibiotics, whereas viral infections are primarily treated using antiviral or anti-inflammatory agents.

Unnecessary antibiotics can be harmful and contribute to microbial resistance, especially in the pediatric population. A rapid and cost-effective diagnostic

(the design concept illustrated in Figure 2C3) [92]. Although this device was only used to distinguish between malaria and dengue fever via simultaneous detection of malaria histidine-rich protein 2, malaria *Plasmodium* lactate dehydrogenase, and dengue nonstructural protein 1 type 2, it was the first multiplex 3D microfluidic POCT device that could successfully differentiate between infectious diseases. Because this multiplex device demonstrated detection accuracy equal to monoplex testing, we suggest further studies to test the previously described biomarker sets in this multiplex 3D microfluidic format to differentiate between bacterial and viral infections in a point-of-care setting. Genetic-based biomarker detection via multiplex microfluidics is usually combined with PCR or loop-mediated isothermal amplification (LAMP) techniques to simplify normally complex operations. LAMP is a novel point-of-care nucleic acid isothermal amplification technique that uses a set of 4–6 primers to recognize 6–8 distinct sequences of target DNA, and can facilitate the creation of a sensitive, rapid, and simple instrument. Multiplex microfluidic LAMP has been used in POCT to differentiate between several types of bacteria and viruses by amplifying and detecting their specific DNA sequences [93–95]. However, gross differentiation of bacterial and viral infection based on this technique has not yet been developed. We suggest further studies to combine multiplex microfluidic LAMP with previously described bacteria/viral differential gene expression signatures.

Microarrays

Microarrays are suitable for high-throughput analysis, which makes them useful for multiplex detection. In one study, a photonic biosensor microarray that could perform label-free detection was used to detect sepsis using protein biomarkers such as CRP, IL-6, and gene biomarkers such as mRNA-16 in whole blood. This biosensor employed a light beam passing through a microarray chip containing the test sample. The resulting changes in phase were recorded using a camera and processed by an algorithm to determine the optical path difference [96]. Although promising, the detection limit of this tool requires further improvement and further clinical testing is warranted.

In addition to previously described genome-based multiplex POCT, clustered regularly interspaced short palindromic repeats (CRISPR)-based nucleic acid diagnostics have recently garnered great attention. CRISPR sequences are bacterial genome arrays that are detectable in the body following viral infections. These sequences are generally derived from DNA fragments of bacteriophages. CRISPR and CRISPR-associated protein (Cas) were first employed for genome editing, but are currently used for genome-based diagnosis of infection and cancer [97]. A commercially available CRISPR/Cas-based platform, named specific high-sensitivity enzymatic reporter unlocking (SHERLOCK), has been developed for use as a multiplex POCT device for genome detection [98]. This can provide highly sensitive and specific results within 1 hour. Furthermore, CRISPR/Cas techniques can be used to create small, inexpensive, amplification-free genome-based POCT [99]. This approach has been used to detect Zika virus, dengue virus, Ebola virus, and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) by targeting the viral genome with high accuracy compared to PCR-based results [100–102]. Moreover, the genome-guiding capacity of CRISPR/Cas allows it to be used as both a sensitive diagnostic tool and an element of antiviral strategies [103]. This technology, as well as recent developments in multiplex POCT devices, target specific infectious pathogens or diseases to facilitate multiplex POCT to discriminate between viral and bacterial infections.

From Basic Research to Clinical Application – The Major Concerns

Distinguishing between bacterial and viral infections is the most effective guide for treatment–antibiotics for bacterial infection and steroid-based/antiviral treatment for viral infection. However, it should be noted that other sources of infection that are not detectable using the proposed

tool is necessary to guide clinical treatment choices.

Current clinically used biomarkers including CRP and PCT are not sufficiently precise to distinguish between bacterial and viral infections. This has led to the characterization of a variety of novel biomarkers, including protein-based biomarkers and gene expression signatures.

In addition to accurate biomarkers, a suitable diagnostic device that corresponds to real clinical scenarios is also required. In common clinical scenarios, sick children are typically brought to clinics where sophisticated analytical devices can be employed. This can result in time delays and significant costs. Point-of-care testing (POCT) is timely and inexpensive and can be used to discriminate between bacterial and viral infections. POCT can provide rapid, easy-to-handle, cost-effective, and convenient biochemically based information, comparable to a pregnancy test.

Combined assays using multiplex POCT techniques and groups of protein biomarkers or streamlined gene expression signatures may provide adequate differential pathogen discrimination.

A clinically applicable diagnostic tool for bacterial and viral infections can promote the quality of care for vulnerable children, reduce global antibiotic overuse, and combat the antibiotic resistance crisis.

protein- and gene-based biomarkers, including fungal, parasite, and prion infections, would require separate differential diagnosis. In addition, because of the high prevalence of infectious disease, it is important to develop clinically applicable tools that can identify the infectious agent and provide rapid, robust, and cost-effective POCT devices. Before a novel biomarker device can be used clinically, however, it should undergo large-scale clinical validation covering a variety of infectious states and types including coinfection. Testing of such devices would require longitudinal follow-up data to determine the optimal diagnostic test window, and interference tests to measure the influence of antibiotics or other drugs on biomarker performance. Given sufficient clinical data to support the use of such tools, clinicians may be able to provide accurate and appropriate treatments, and also reduce global antibiotic overuse.

Concluding Remarks

In recent decades, substantial progress has been made towards the development of host-derived biomarkers for discriminating between bacterial and viral infections in pediatric populations. This evolution includes the discovery of novel protein biomarkers and biomarker combinations. In addition, a series of gene expression signatures have also been discovered that have promise in differentiating between bacterial and viral infections. Further, recent studies have identified a small set of novel biomarkers that may be useful for clinical application. Note, most of the novel diagnostic biomarkers discussed here have only been evaluated in single studies, which prompts questions regarding their reliability (see Outstanding Questions). In addition, there are concerns regarding the cost–performance ratio and the analytical accessibility of these biomarkers. When used in combination, protein biomarker sets and host gene expression signatures could provide accurate information. Integrated into multiplex POCT diagnostic devices, these will enable rapid and cost-effective pathogen discrimination to provide accurate treatment guidance. Rapid progress in this field is expected to improve pediatric care and further reduce global antibiotic overuse.

Supplemental Information

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