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REVIEW ARTICLE

Advances in Spectral Techniques for Detection of Pathogenic Microorganisms

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Abstract

The highly contagious viral illness Coronavirus disease 2019, caused by severe acute respiratory syndrome coronavirus-2, has led to nearly 5 million deaths worldwide. The detection of highly infectious pathogens or novel pathogens causing emerging infectious diseases is highly challenging. Encouragingly, spectral detection—including laser-induced fluorescence spectroscopy, infrared absorption spectroscopy, Raman spectroscopy and their combinations—has been broadly used to detect pathogenic microorganisms on the basis of their physical and chemical characteristics. Surface-enhanced Raman spectroscopy with labels can detect organisms at a minimum concentration of 3 cells/mL. The changes in cells' biochemical reactions before and after polioviral infection can be detected by Fourier transform infrared spectroscopy. However, the sensitivity and specificity of different spectral detection categories differs, owing to their different detection principles. Flexible detection methods require interdisciplinary researchers familiar with both pathogen biology and instruments. This review summarizes the advances in spectral techniques used in detecting pathogenic microorganism.

Key words: pathogenic microorganism detection, laser-induced fluorescence spectroscopy (LIFS), infrared absorption spectroscopy (IRAS), Raman spectroscopy (RS), Fourier transform infrared (FTIR)

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INTRODUCTION

Infectious diseases caused by pathogenic microorganisms, including bacteria, fungi, viruses and protozoa, remain a major concern because they pose severe threats to public health [1,2]. Diverse pathogen transmission routes and carriers greatly increase the difficulty in detection, and increase the workload and financial burden required for prevention and control [3,4].

In the past 2 years, Coronavirus Disease 2019 (COVID-19) infections have rapidly developed into a pandemic, causing more than 356 million confirmed cases and 5.61 million deaths as of 26 January 2022 [5]. At present, the most widely used methods are nucleic acid amplification testing (NAAT) and serological detection [6]. Traditional microbiological testing methods (such as morphological diagnosis, selective medium cultivation and identification medium cultivation) are labor-intensive and time-consuming [7]. To achieve more convenient and fast detection, new spectroscopy technologies using lasers, computer information processing and nanomaterials have been developed [8,9]. Spectroscopy can be used for the qualitative and quantitative assessment of detected pathogenic microorganisms according to the specific spectra emitted by the surface molecules [10,11]. Among the many spectral

detection methods, fluorescence spectroscopy, infrared (IR) spectroscopy and Raman scattering spectroscopy have broad application prospects because of their high sensitivity and resolution, wide linear range and absence of sample destruction [12-14]. Here, we review the application of these three spectral technologies in the detection of pathogenic microorganisms, and compare the advantages and disadvantages of each method.

LASER-INDUCED FLUORESCENCE SPECTROSCOPY

Laser-induced fluorescence spectroscopy (LIFS) involves irradiating the sample with a laser and recording the specific fluorescence emitted [15]. Because the residues have different molecular structures, such as double bonds, amino acids and benzene rings, they emit different fluorescence under the excitation of visible or ultraviolet (UV) light. Many typical molecules, such as tryptophan, tyrosine, phenylalanine and nicotinamide adenine dinucleotide (NADH), emit specific fluorescence after laser excitation, thus allowing characteristic fluorescence spectra to be obtained and enabling pathogenic microorganisms to be identified according to their molecular structures [16,17]. Because the laser parameters can be precisely controlled, and the photodetector is highly sensitive to fluorescence signals, LIFS has gained increasing attention and application in pathogen detection.

LIFS in bacterial detection

Studies have shown that the maximum excitation wavelength of amino acids is 250–280 nm, and that of NADH is 310–340 nm. In one study, different laser excitation wavelengths (250, 270 or 316 nm) were used to excite 25 bacterial suspensions and distinguish them according to the large variations in their fluorescence spectra resulting from their surface residues [18]. Principal component analysis (PCA) and hierarchical clustering analysis (HCA) were then used to verify the spectral results, which distinguished these 25 bacterial samples at the strain level. By using LIFS, six microorganisms (details in Table 1) from a variety of interfering air substances have been quickly distinguished in several minutes [19]. The fluorescence emission intensity of the microorganisms decreases, and the position of the main peak shifts according to the different cell components. In addition, many studies have confirmed that fluorescence LIDAR can detect the presence of specific bacteria in bioaerosols, thus aiding in monitoring of hazardous biological agents [16,20,21]. The spores of *Bacillus anthracis* have been detected by fluorescence LIDAR with different excitation wavelengths, and a wavelength of 294 nm instead of 355 nm has been found to decrease the error rate and increase the sensitivity of identification [21].

LIF bacterial detection has also been used in environmental pollution and food safety applications. A hand-held fluorescence spectrometer with a double monochromator has been designed to detect *Escherichia coli* and *Bacillus* contamination in laboratory settings [22]. This device can measure the proportion of live/dead bacteria after UV radiation and provide information on the treatment efficacy of bacterial infections [23]. A hand-held device has also been developed to detect the microorganisms on equipment surfaces in hospitals, according to the dual-peak fluorescence of tryptophan [24]. This device enables the acquisition of more comprehensive excitation-emission matrix data, such that a three-dimensional data-cube fluorescence spectrum can be obtained for each sample. Moreover, this device is easy to operate without professional training and therefore is suitable for use in clinical settings. A UV-LIF instrument with a pulsed 355-nm laser has been used to detect *Bacillus thuringiensis* in the presence of pollen interference; this device decreases computation time through gaining more photons in each channel by sacrificing some sensitivity without influencing discrimination among analytes [25]. LIFS together with PCA has been used for the rapid detection of lactic acid bacteria and several pathogenic bacteria, such as *Salmonella* and *Campylobacter* [26,27].

LIFS in fungal detection

Fungal infection can lead to tinea pedis, tinea manuum, respiratory allergic reactions or pulmonary fungal infections causing severe respiratory failure and death [28]. Compared with the previous generation of bioaerosol sensors, the waveband integrated bioaerosol sensor has two pulsed xenon UV sources that emit fluorescence from bioaerosols [29]. Combined with various measurement parameters, such as particle size, asymmetry and automatic fluorescence, this sensor better separates the two detection bands and matches the peak emission bands of tryptophan and NAD(P)H. This instrument's optics configuration and optical filters have been modified to distinguish fungal spores from pollen. Other modified instruments, such as a UV aerodynamic particle sizer and dual-excitation-wavelength particle fluorescence spectrometer, have also been applied to detect biological aerosols [17,30-32]. Microorganisms can be identified on the basis of the strong presence of tryptophan emission in the 350 nm band under 266 nm laser excitation, or without fluorescence emission under 355 nm laser excitation. On the basis of this phenomenon, a two-wavelength (266 nm and 355 nm) LIFS instrument has successfully distinguished diesel soot, fungi and bacteria, thus overcoming the deficiencies of single wavelength LIFS instruments [33].

LIFS in viral detection

A new LIFS device developed to monitor microbial contamination in a real-time mode, has successfully distinguished bacteria, bacterial spores, fungal conidia and viruses by assessing their spread concentrations [34]. The tryptophan in the viruses phi6 and phi12, and their bacterial host proteins is the predominant fluorophore under UV light, and the fluorescence characteristics of tryptophan vary according to the protein environment [35]. Combined with PCA, a 266 nm UV laser has been used to discriminate different viruses on the basis of the wavelength signals from envelope proteins [36]. However, owing to the limits of the

TABLE 1 | Current LIFS technology applications for pathogen detection.

Detected pathogens	Targets and criteria for detection	Excitation wavelengths	Conclusion (discrimination/sensitivity/specificity)	References
Lactococcus lactis *Pseudomonas pentosaceus* *Kocuria varians* *Pseudomonas fluorescens* *Lactococcus innocua* *Lactobacillus curvatus* *Lactobacillus farciminis* *Lactobacillus pentosus* *Lactobacillus viridescens* *Lactobacillus bavaricus* *Lactobacillus plantarum* *Lactobacillus sakei* *Staphylococcus xylosus* *Staphylococcus carnosus* *Staphylococcus saprophyticus* *Staphylococcus warneri* *Staphylococcus caprae* *Staphylococcus haemolyticus* *Staphylococcus aureus* *Staphylococcus epidermidis*	**AAA+NA, tryptophan and NADH, PCA and Mahalanobis distances**	250, 270 or 316 nm	① *Lactococcus lactis*, *Pseudomonas pentosaceus*, *Kocuria varians*, *Pseudomonas fuorescens* and *Lactococcus innocua* can be well distinguished. ② *Lactococcus*, *Pediococcus*, *Kocuria*, *Pseudomonas* and *Listeria* can be well distinguished (calibration spectra, 99.2% discrimination; validation spectra, 80%). ③ *Staphylococcus* species can be well distinguished (calibration spectra, 100% discrimination; validation spectra, 82/87.5%). ④ *Lactobacillus* species can be well distinguished (calibration spectra, 100% discrimination; validation spectra, 81%).	[18]
Staphylococcus aureus *Escherichia coli* *Pseudomonas aeruginosa* *Bacillus subtilis* *Candida albicans* *Aspergillus niger*	**NADH, riboflavin, dipicolinic acid, PCA, HCA**	405, 440 or 470 nm	The fluorescence spectra of microorganisms change significantly (fluorescence intensity decrease ≥30%; main peak shift ≥2%) when illuminated by laser at a concentration of 10^3 cfu/L.	[19]
Anthrax simulants	**NADH, tryptophan, tyrosine**	294 or 355 nm	Excitation at 294 nm makes classification more accurate, with ~7 nm spectral resolution compared with 355 nm.	[21]

TABLE 1 | Continued

Detected pathogens	Targets and criteria for detection	Excitation wavelengths	Conclusion (discrimination/sensitivity/specificity)	References
Escherichia coli *Bacillus*	**Tryptophan, tyrosine, DNA**	270 nm	A hand-held synchronous scan spectrometer can discriminate live/dead bacteria at concentrations of 10^8–10^2 cells/mL within 10 min.	[22,23]
Staphylococcus aureus *Staphylococcus carnosus* *Clostridium difficile* strain CD630 *Clostridium difficile* strain *Klebsiella pneumoniae* *Serratia marcescens* *Proteus mirabalis* *Pseudomonas aeruginosa* *Escherichia coli*	**Tryptophan, NAD(P)H**	220 or 280 nm	With the excitation source at 280 nm, bacterial contamination can be detected in most healthcare-associated materials at 10^7–10^8 cells/mL at an emission peak of 340 nm.	[24]
Bacillus thuringiensis *Bacillus atrophaeus*	**-**	355 nm	A total of 10–20 spectral bands are sufficient to distinguish *Bacillus thuringiensis*, *Bacillus atrophaeus*, ovalbumin and pollen in concentrations of approximately 5000–20000 ppl (particle concentration).	[25]
Lactic acid bacteria	**Aromatic amino acids, nucleic acids, NADH, FAD**	250, 316 or 380 nm	A total of 29 wild strains of lactic acid bacteria can be discriminated between *Lactobacillus sakei* subsp. *carnosus* and *Lactobacillus sakei* subsp. *sakei* by three excitations with high classification accuracy (98.3% in aromatic amino acid and nucleic acid spectra; 88.9% in NADH spectra; 88.9/97.8% in FAD spectra).	[26]
Escherichia coli *Salmonella* *Campylobacter*	**PCA**	225 or 280 nm	The optimum excitation and emission wavelengths were found with a synchronous scan technique and detected in three bacterial samples with a limit of 10^3–10^4 cells/mL.	[27]
Aspergillus fumigatus *Cladosporium cladosporioides* *Cladosporium herbarum* *Alternaria alternata* *Pencillium notatum*	**Tryptophan, NAD(P)H**	280 or 370 nm	By recording more than 2000 individual-particle measurements, waveband integrated bioaerosol sensor-4 can distinguish natural airborne primary biological aerosol particulate samples and toxic fungal spores in real-time.	[29]
Yersinia rohdei MS2 (bacteriophage) in *Escherichia coli* lysate	**NADH, tryptophan**	263 or 355 nm	Humidity up-regulation and ozone exposure decrease the intensity and position (blue-shifted) of the fluorescence emission peak, and could be applied in detecting oxidation of biological particles.	[31]

TABLE 1 | Continued

Detected pathogens	Targets and criteria for detection	Excitation wavelengths	Conclusion (discrimination/sensitivity/specificity)	References
Sporisorium Cruentum	**Two excitation wavelengths**	266 or 355 nm	Two 266 nm excitation beams separated by 200 ns increase discrimination among individual laboratory-generated aerosol particles. Bacterial sample particles can be better discriminated from diesel engine particles by comparison of the ratio of the 450 nm band excited by 266/355 nm.	[33]
Bacillus atrophaeus (biological warfare agent simulants)				
Globigii (biological warfare agent simulants)				
Bacillus subtilis				
Brucella neotomae				
Escherichia coli	**Tryptophan, tyrosine, phenylalanine, NADH**	248 or 351 nm	A dual wavelength LIF device can detect *Escherichia coli* and MS2 viruses with a LOD of 2.9×10^4 and 9.5×10^4 cfu/cm^2, respectively.	[34]
Bacillus thuringiensis				
Cladosporium herbarum				
MS2 (bacteriophage)				
Cystovirus infected *Pseudomonas syringae*	**Tryptophan**	340 nm	The ratio of fluorescence amplitudes of 331 nm and 344 nm detected by LIFS can be used to characterize the viral infection process.	[35]
Hepatitis A	**PCA**	266 nm	LIFS is suitable for the detection of viruses in environmental samples and can detect hepatitis A at concentrations between 2×10^4 and 2×10^5 TCID50/mL.	[36]
Coxsackie A7				
Coxsackie A9				
Coxsackie B4				
Rotavirus				
Echovirus type 1				
Astrovirus				

Abbreviations: PCA (principal component analysis), NADH (nicotinamide adenine dinucleotide), NADPH (nicotinamide adenine dinucleotide phosphate), HCA (hierarchical clustering analysis), FAD (flavin adenosine dinucleotide), LOD (limit of detection), TCID (tissue culture infective dose).

biological structures and featureless spectral peaks of viruses, virological analysis is time-consuming and expensive. LIFS has been gradually improved to provide more accurate excitation laser and classification techniques, thus promoting viral monitoring and epidemiologic studies.

INFRARED ABSORPTION SPECTROSCOPY

IR absorption spectroscopy (IRAS) is based on the absorption of certain wavelength IR rays after the material molecules are irradiated by changing frequencies of IR light [37]. Owing to the different compositions and vibration modes of molecular groups, IRAS can be divided into a characteristic frequency region and fingerprint region, which are used to classify and identify pathogenic microorganisms [38]. The characteristic frequency region is produced mainly by the stretching vibration of the specific group, and can be used to identify functional groups. The fingerprint region is generated by the stretching vibration of some single bonds, such as C-O and C-N, and the bending vibration of hydrogen-containing groups such as the C-H and C-C skeleton. The IR spectral ranges of different microorganisms are listed in Table 2.

According to the principle of IRAS, Fourier transform IR spectroscopy (FTIR) uses an additional component called an interferometer to obtain the interferogram of a sample. The detector and computer then convert the interferogram into data, which are transformed into an actual spectrum through Fourier mathematical transformation [39]. FTIR not only reflects the molecular vibration information of mixed components—such as proteins and nucleic acids in the cell wall, cell membrane, and even the nucleus in microorganisms—but also can sensitively detect changes in molecular groups and their surrounding environment, thus enabling the types and states of microorganisms to be distinguished and identified.

FTIR for bacterial detection in food

FTIR is commonly used for general determination and has been confirmed to provide rapid and accurate results for the identification of pathogenic microorganisms in food [40]. FTIR can be combined with multivariate analysis to identify organisms at the species and strain levels, thus helping to prevent food poisoning and zoonoses [41,42]. Through a combination of FTIR with machine learning methods such as PCA, 24 strains in five species of *Pectobacterium* and *Dickeya* have been identified with 99% accuracy at the genus and species levels, and above 94% accuracy at the strain level [43]. A total of 174 food-related bacteria (of 93 species) from an in-house collection and 40 fresh isolates from routine foods have been analyzed by FTIR and matrix assisted laser desorption/ionization-time of flight mass spectrometry, with identification accuracy at the species level of 88% and 75%, respectively [44]. When combined with an artificial neural network (ANN), FTIR has higher reliability at the species level. In one study, detection of probiotics in animal provender has identified 92 lactic acid bacteria species out of nine genera and strains, with 93.2% accuracy at the species level and 97.1% accuracy at the strain level [45].

Application of FTIR for bacterial detection in clinical settings and of zoonoses

Accurate detection of clinical pathogens can help physicians use antibiotics rationally and control the abuse of broad-spectrum antibiotics. FTIR has been proposed for the typing of clinical *Enterobacter cloacae* complex isolated with whole genome sequencing; it has also been found to aid in determining whether the isolates are clone-related, thus serving as a powerful tool for outbreak investigation and analysis [46]. Through a combination of FTIR with ANN, *Staphylococcus aureus* serotypes CP5 and CP8 have been successfully identified [47]; this method could be applied to the diagnosis and large-scale epidemiological monitoring of *Staphylococcus aureus*. On the basis of the molecular structure of lipids, proteins and nucleic acids, FTIR has been used in mid-IR spectroscopy (4000–400 cm^{-1}) to detect urinary tract infection (UTI) in urine specimens. Therefore, FTIR can be considered a supplementary method for urine sediment examination and for the detection of pathogenic bacteria in UTI [48]. FTIR has also been used to classify tested bacteria into sensitive and resistant types, thus avoiding unnecessary costs and adverse effects due to excessive treatment or treatment errors [49,50].

Graphene oxide (GO)-based materials are widely used in FTIR because they contain many oxygen-bearing groups, thus enabling ligand conjugation at high density and increasing the sensitivity of detection [51]. GO combined with FTIR spectroscopy has been used to detect *Escherichia coli*, and the IR peaks have been found to significantly shift after *Escherichia coli* interaction with antibody-coated GO-nano-biosensor units with respect to a negative control [52]. FTIR combined with immunomagnetic separation has been found to be highly sensitive in identification of *Salmonella typhimurium* [53]. FTIR combined with cellular-based sensing technology has been used to compare the cell biochemical reactions before and after polioviral infection through absorbance changes, thus suggesting a more automated approach for viral detection [54].

The presence of *Escherichia coli* and *Cryptobacillus pyogenes* in Argentine cow uterine specimens has been detected by FTIR, thus simplifying the identification of uterine pathogens and increasing treatment efficiency [55]. FTIR has successfully identified *Trueperella pyogenes* isolated from clinical mastitis in dairy cows, and can be used in phenotyping and genotyping [56].

Application of ATR-FTIR in multiple pathogen detection

Attenuated total reflection FTIR (ATR-FTIR) applies attenuated total reflection technology to FTIR [57]. It can directly obtain structural information on organic components through the reflection signal of the sample surface, and has been widely used for detection in food, environmental and drug samples [58-60]. Because of its simplified sample processing steps, stability and high sensitivity, ATR-FTIR has been widely used for fungal detection, to enable timely identification of mold contamination to avoid food-borne

TABLE 2 | Pathogens detected by FTIR/ATR-FTIR, specific detection/analysis methods and applications.

	Detected pathogens	Spectral range	Auxiliary detection method	Application	References
FTIR	*Vibrio parahaemolyticus*	Between 1800 and 900 cm^{-1}	PCA, HCA	Subtyping pathogenic and non-pathogenic *Vibrio parahaemolyticus*	[41]
	Pseudomonas aeruginosa *Pseudomonas fluorescens* *Pseudomonas litoralis* *Pseudomonas putida* *Escherichia coli* *Escherichia fergusonii* *Escherichia hermannii* *Bacillus cereus* *Bacillus thuringiensis*	Between 4000 and 400 cm^{-1}	HCA	New bacterial identification method	[42]
	Pectobacterium parmentieri *Pectobacterium carotovorum subsp. brasiliense* *Pectobacterium carotovorum subsp. carotovorum* *Dickeya solani* *Dickeya Dianthicola*	Between 900 and 1800 cm^{-1}	Liquid nitrogen-cooled mercury cadmium telluride detector, PCA, ANN, polynomial support vector machines	Differentiation of *Pectobacterium* and *Dickeya* spp. phytopathogens	[43]
	Bacillus cereus *Escherichia coli* *Pseudomonas aeruginosa* *Staphylococcus aureus* *Staphylococcus epidermidis*	Between 4000 and 700 cm^{-1}	MALDI-TOF HCA, ANN	Microbial identification in the food industry	[44]
	92 Lactic acid bacteria species	Between 700–1800 cm^{-1} and 2800–3000 cm^{-1}	ANN	Identification of lactic acid bacteria in food at the species and strain levels	[45]
	Enterobacter cloacae	Between 1300 and 800 cm^{-1}	ANN	Tool for strain typing of clinical *Enterobacter cloacae* complex strains.	[46]
	84 Staphylococcus aureus strains	Between 1200 and 800 cm^{-1}	ANN, PCA	Discrimination of *Staphylococcus aureus* capsular serotypes	[47]
	Urine samples from patients with pyuria and hematuria *Staphylococcus aureus* *Escherichia coli* *Klebsiella pneumonia* *Pseudomonas aeruginosa* *Enterococcus faecalis*	Between 4000 and 400 cm^{-1}	PCA, MALDI-TOF	Urine sediment examination and detection of pathogenic bacteria in UTI	[48]
	Escherichia coli	Between 900–1795 cm^{-1} and 2800–3000 cm^{-1}	MALDI-TOF	Determination of bacterial drug resistance	[49]

TABLE 2 | Continued

	Detected pathogens	Spectral range	Auxiliary detection method	Application	References
	Candida albicans	Between 4000 and 900 cm^{-1}	-	Determination of *Candida albicans* drug resistance	[50]
	Escherichia coli	Between 4000 and 500 cm^{-1}	Graphene oxide-coated polycarbonate track-etched platform	Real-time *Escherichia coli* detection with a low limit of detection	[51,52]
	Salmonella Typhimurium *Klebsiella pneumoniae* *Salmonella serovars* *Escherichia coli* *Proteus mirablis* *Citrobacter freundii* *Pseudomonas aeruginosa*	Between 4000 and 400 cm^{-1}	Immunomagnetic separation	*Salmonella* identification in foods	[53]
	Poliovirus PV1	Between 3600 and 650 cm^{-1}	-	Infective virus particle detection	[54]
	Escherichia coli *Trueperella pyogenes*	Between 4000 and 500 cm^{-1}	ANN, PCA	Database for *Escherichia coli* and *Trueperella pyogenes* detection	[55,56]
ATR-FTIR	*Alternaria cucumerina* *Alternaria brassicicola* *Cladosporium cucumernum* *Corynespora cassiicola* *Botrytis cinerea* *Trichothecium roseum* *Fusarium oxysporum* *Fusarium solani* *Fusarium semitectum* *Myrothecium roridum* *Pestalotiopsis guepinii* *Colletotrichum orbiculare* *Phomopsis vexans* *Ascochyta citrullina* *Rhizoctonia solani* *Pythium aphanidermatum*	Between 1800 and 900 cm^{-1}	Clustering analysis	Accurate classification of plant pathogenic fungi at the species level	[61]
	Aspergillus ochraceus *Aspergillus niger* *Candida glabrata* *Penicillium roguefortii*	Between 900 and 700 cm^{-1}	PCA, canonical variate analysis	Household fungal detection with optimum classification	[62]
	Aspergillus flavus *Aspergillus parasiticus* *Aspergillus ochraceus*	Between 4000 and 600 cm^{-1}	PLSR	Moldy peanut detection	[64]

TABLE 2 | Continued

Detected pathogens	Spectral range	Auxiliary detection method	Application	References
Fusarium oxysporum *Penicillium aurantiogriseum* *Penicillim expansum* *Aspergillus glaucus* *Aspergillus candidus*	Between 4000 and 800 cm^{-1}	PCA, PLS discriminant analysis	Rapid identification of bacterial molds of different genera	[65]
Staphylococcus aureus	Between 4000 and 650 cm^{-1}	PCA, PLS discriminant analysis	Detection of low-level vancomycin-resistant *Staphylococcus aureus*	[66]
Begomoviruses	Between 4000 and 400 cm^{-1}	PCA, PLS discriminant analysis	Detection of begomoviral infection in papaya plants	[67]

Abbreviations: PCA (principal component analysis), NADH (nicotinamide adenine dinucleotide), NADPH (nicotinamide adenine dinucleotide phosphate), HCA (hierarchical clustering analysis), FAD (flavin adenosine dinucleotide), PLSR (partial least squares regression), PLS (partial least squares), ANN (artificial neural network), MALDI-TOF (matrix-assisted laser desorption/ionization mass spectrometry).

poisoning and facilitate early treatment. Through ATR-FTIR spectroscopy, IR spectra with high resolution and good reproducibility have been obtained for the classification of 17 fungal strains from 14 genera, with a sensitive spectrum region of 1800–900 cm^{-1} [61]. Four household fungi (*Aspergillus ochraceus*, *Aspergillus niger*, *Candida glabrata* and *Penicillium roguefortii*) have been distinguished by ATR-FTIR with a sensitive spectrum region of 900–700 cm^{-1}; however, the spectral results combined with chemometric analysis resulted in 100% accurate classification and therefore may serve as a promising diagnostic tool for household fungi [62].

Partial least squares regression (PLSR) models, tools for multivariate statistical analysis, have been combined with ATR-FTIR to detect aflatoxin in feed containing moldy peanuts [63,64]. ATR-FTIR and PLSR models have been expanded to the detection of *Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus ochraceus* after various peanut storage periods [64]. ATR-FTIR and chemometrics have been used to rapidly identify seven fungal strains in the 1000–900 cm^{-1} spectrum region, thus indicating that ATR-FTIR is feasible for rapid fungi identification in paddy rice [65].

In addition to fungal identification, ATR-FTIR has been applied in bacterial and viral detection. Heterogeneous vancomycin-intermediate *Staphylococcus aureus* (hVISA) with diminished sensitivity to vancomycin poses many difficulties in clinical therapy. Therefore, hVISA must be differentiated from vancomycin-susceptible *Staphylococcus aureus* isolates. With ATR-FTIR, 59 clinical methicillin-resistant *Staphylococcus aureus* isolates have been found to have different absorption bands (1087 and 1057 cm^{-1}), and this method has been found to efficiently distinguish hVISA and vancomycin-susceptible *Staphylococcus aureus* isolates on the basis of the cell wall content [66].

In virus infected papaya trees, ATR-FTIR has successfully revealed a unique peak representing viral infection at 2391.54 cm^{-1}. Therefore, ATR-FTIR can be applied to asymptomatic plant viral detection, providing the advantages of sensitivity and avoiding the tedious pretreatment process [67].

RAMAN SPECTROSCOPY

Raman spectroscopy (RS) emerged after the discovery of the Raman effect in 1928. This non-destructive chemical analytic technique provides both qualitative and quantitative molecular information, according to the fundamental vibrational modes of molecules [68]. RS is performed by exciting a sample with a laser and measuring the inelastic scattering of photons from the vibrations within the molecules [69,70]. In recent years, RS has been continually expanded with the rapid development of emerging nanomaterials, thus providing new technologies such as resonance Raman spectroscopy (RRS), confocal microprobe Raman spectroscopy (CMRS) and surface-enhanced Raman spectroscopy (SERS).

RRS in multiple pathogen detection

RRS has a laser excitation frequency closer to the electronic transition of samples, thus enhancing the Raman scattering intensity by a factor of 10^2–10^6 and improving the signal-to-noise ratio [71]. RS and coherent anti-Stokes Raman scattering microscopy have been used to observe the distribution of cytochromes in hyphal tip cells of *Schizophyllum commune*, detecting fungal mycelial mitochondria in a label-free manner [72]. An excitation maximum at 555 nm results in solid state autofluorescence in the condensed crystalline phase of iron (III) protoporphyrin IX isolated from synthetic heme anhydride, which can be applied in parasite determination and drug screening [73]. In addition, there is an aggregation enhancement effect at 1372 cm^{-1} (for excitation wavelengths 568 nm and 830 nm) attributed to the overlap of two hematins [74].

UV-RRS can enhance the Raman spectra of more characteristic biological targets such as proteins; in contrast, DNA is not enhanced, thus resolving the problem of fluorescence interference in RRS [75]. UV-RRS has successfully detected *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis* and *Enterococcus* spp. at 1475 cm^{-1} and 1600 cm^{-1} characteristic peaks in samples of 20 UTI clinical isolates [76].

CMRS

SERS has been widely used to identify and characterize microorganisms, but its small scattering cross section perturbs signals in bulk particle suspension measurements [77]. CMRS can overcome these drawbacks, mainly by probing small volumes with a focused excitation laser beam and imaging the collected scatter through a matched aperture [78]. CMRS combines the advantages of RS and microscopy in that it is fast, non-destructive, requires small samples and does not require pretreatment, in contrast to conventional RS. Furthermore, it can acquire signals of microscopic objects at high spatial resolution (<1 μm) with a high-power optical microscope.

CMRS has been used to localize the aerial hyphae of *Colletotrichum camelliae* Massee in vivo according to a fingerprint band at 1622 cm^{-1} corresponding to chitin [79]. Compared with traditional RS, this method can avoid the tedious process of making slices as well as the shortcomings of loss of fungal activity because of the use of chemical reagents. CMRS has also been applied to identifying parasites, such as in malarial pigment detection, in which an additional band is detected at 1655 cm^{-1} in the spectra of hemozoin and beta-hematin in addition to hematin and hemin [80]. Furthermore, a characteristic peak detected at 1372 cm^{-1} can be used for determining malarial pigment [81].

SERS in multiple pathogen detection

As early as 1974, pretreatments of roughened silver electrode were used to improve adsorption effect of pyridine molecules, which significantly increased the Raman signal of pyridine intensity [82]. Until 1977, SERS was defined according to the enhancement associated with the rough gold and silver surfaces [83]. Through coupling of noble metal nanoparticles (NPs), the SERS signal of some fluorescent dye molecules is enhanced by 10^{10}–10^{14} times that with ordinary RS [84,85].

The main SERS peaks are derived from nucleic acids, proteins, polysaccharides, carbohydrates and lipids from bacterial cell walls [86]. The spectra of foodborne and waterborne bacteria (*Escherichia coli* O157: H7, *Staphylococcus epidermidis*, *Listeria monocytogenes* and *Enterococcus faecalis*) show significant differences in the Raman shift region between 500 and 1800 cm^{-1}: bands at 928 cm^{-1} and 1101 cm^{-1} appear for only *Staphylococcus epidermidis* and *Enterococcus faecalis*, but not *Listeria monocytogenes*. Interestingly, *Staphylococcus epidermidis* shows stronger absorption than *Escherichia coli* O157:H7 at the 1334 cm^{-1} band, owing to the different ring stretching modes of guanine and adenine [86].

SERS detection of pathogenic microorganisms can be divided into label-free and label methods. Label-free SERS directly attaches bacteria to the SERS solid substrate or the SERS active NP substrate in a solution; in contrast, label SERS connects a Raman reporter to the surfaces of plasma resonance Ag or Au NPs, and further modifies them with specific antibodies or aptamers of pathogens as recognition molecules to create SERS tags that yield enhanced Raman signals [87].

Label-free SERS in bacterial detection

Two common substrates are applied in label-free SERS: nanostructured metal surfaces and noble metal NPs. Label-free SERS is commonly used to detect specific molecules on bacterial surfaces [88].

A. SERS based on nanostructured metal surfaces. An Ag nanocrystal substrate has been explored for the identification of pathogens including *Escherichia coli* O157, *Salmonella typhimurium* and *Staphylococcus aureus*, as well as live and dead bacteria [89]. A SERS substrate composed of unique quasi-3D plasmonic nanostructure arrays has been developed to detect seven strains of the marine pathogen *Vibrio parahaemolyticus* [90]. However, these two SERS nanostructured metal surface substrate only improve the detection sensitivity, but cannot accurately quantify the results. With silver particles as a SERS substrate, Gram-positive bacteria form a peak at 497 cm^{-1} representing cell wall polysaccharides, thus enabling differentiation from Gram-negative bacteria [89,90]. SERS with vancomycin coated silver nanorod substrates has also been used to detect 27 different bacteria, distinguishing Gram-positive from Gram-negative bacteria according to the intrinsic structural differences in the cell walls [91].

B. SERS based on noble metal NPs. NPs can be deposited on bacterial cell walls through electrostatic attraction, owing to the presence of lipopolysaccharide or teichoic acid on the cell walls of both Gram-negative and Gram-positive bacteria [92]. In contrast to the long preparation process and high cost of solid substrates, preparation and synthesis are easy for noble metal NPs, which therefore are more widely used. With multivariate analysis and an enhanced substrate of gold nanorods, *Pseudomonas* spp. have been classified [93]. However, the steps described above are complicated and

cannot support quantitative analysis, whereas in situ synthesis of NPs ensures homogeneous contact to bacterial cell walls. Through electrostatic interaction, Ag NPs can be synthesized in situ on bacterial cell walls for quantitative detection and identification of pathogens [94,95]. Moreover, microarrays can also be used for direct in situ synthesis of Ag NPs on bacterial surfaces, and can ultimately quantitatively distinguish live from dead bacteria [96].

However, the application of label-free SERS methods is substantially limited by the weak detection signal and the indistinguishable peaks, together with the lack of a standard bacterial Raman fingerprint spectral database [94].

Label SERS in bacterial detection

Label SERS can assemble target recognition molecules and a Raman reporter into SERS labeled probes to detect amplified Raman signals from bacterial cell walls or extracellular membranes. Therefore, this method is widely used.

A. Target recognition molecules. Target recognition molecules are used mainly to improve the biocompatibility and targeting function (specific binding of pathogens) of SERS probes.

Antibodies have been widely used as recognition elements, owing to their specificity through covalent binding. Gold nano-popcorn attached single-walled carbon nanotubes conjugated with monoclonal antibodies have been used to detect *Escherichia coli* in water [97]. Au-coated magnetic NPs conjugated with antibodies to *Staphylococcus aureus* have been synthesized to capture and separate bacteria [98]. For detection of *Salmonella choleraesuis* and *Neisseria lactamica*, specific antibodies have been embedded on the surfaces of nanoaggregate-embedded beads and coated with a small number of gold NPs [99].

Beyond antibodies, aptamers—single-stranded DNA or RNA molecules with high binding affinities to specific targets—have been applied to pathogen detection. The synthesis of aptamer-Fe_3O_4@Au magnetic NPs and prepared vancomycin-SERS tags has achieved a detection limit of 3 cells/mL [100]. In a sandwich-like complex based SERS aptasensor approach, Au@Ag-apt1-target-apt2-X-rhodamine has been used for quantitative detection of *Salmonella typhimurium* with a sensitivity of 15 cfu/mL [101].

Similarly to antibodies, phages have high specificity toward targeted bacteria and have been widely applied in bacterial typing and identification. A reproducible SERS nanosensor coated with phages has been designed to rapidly identify bacteria without complex sample preparation [102]. A specific phage against *Escherichia coli* has been designed as a biological probe, which interacts with gold nanorods and gathers around the negatively charged bacterial cell walls; consequently the target bacteria can be detected without use of a SERS substrate [103]. Because phages specifically recognize target bacteria, the SERS spectra of only target bacteria, but not non-target bacteria, change over time.

B. Raman reporter. Labeled SERS probes derive their signals from Raman reporters, usually based on the organic dye molecules containing sulfur or nitrogen, which have high affinity for Ag and Au substrates, and ensure the stability of the Raman probe. Currently, 4-mercaptobenzoic acid (4-MBA), 4-mercaptophenylboronic acid (4-MPBA), 4-aminothiophenol (4-ATP) and 4-mercaptophenol (4-MPh) have been successfully introduced in SERS tag based bacterial assays [97,104–106]. The 4-MPBA probe has been used as an indicator molecule to detect *Escherichia coli*, *Salmonella enteritidis* and *Listeria monocytogenes* on filter membranes in drinking water [107]. A characteristic 4-MPBA signal is observed in the presence but not the absence of bacteria. Nonetheless, the method still has several drawbacks and may not be able to distinguish live from dead bacterial cells [107].

SERS used in SARS-CoV-2 detection

Because only SERS substrate and RS are needed, SERS is considered a fast and feasible method to detect the biochemical structures of viral envelopes [108]. The detection of SARS-CoV-2 with SERS can be completed in 5 minutes, whereas NAAT or serological tests require at least half an hour. Moreover, SERS is uniquely able to detect live and dead SARS-CoV-2 viruses at any stage of infection, whereas NAAT and serological tests are suitable for pre-infection and post-infection, respectively. Recently, a new SERS based aptasensor combining gold nanopopcorn as the substrate and a spike protein deoxyribonucleic acid aptamer as the receptor has shown enhanced sensitivity and specificity in the detection of SARS-CoV-2, with a limit of lower than 10 pfu/mL in 15 min [109]. On the basis of the high binding ability of the spike glycoprotein of SARS-CoV-2 toward the receptor human angiotensin converting enzyme 2 (ACE2), various SERS sensors have been developed to capture SARS-CoV-2. One method uses a silver-nanorod SERS array functionalized with ACE2, whereas another has added a synthesized peptide sequence derived from the ACE2 domain onto the SERS active substrate [110]. The former method has accuracy consistent with that of RT-PCR and avoids the tedious process of RNA extraction; therefore, this method could be used for rapid detection in the field. In addition to being highly selective, the second method can quantify spike protein with a detection limit of 300 nM. Beyond these two methods, a label-free ACE2-functionalized hierarchical gold nanoneedle array has been demonstrated to detect single viruses within 5 min, with a detection limit as low as 80 copies mL^{-1} [111]. Recently, a hand-held SERS-based breathalyzer using breath volatile organic compounds as a COVID-19 biomarker has been developed, which can classify the breath metabolites among infected and non-infected people in less than 5 min through PLS discriminant analysis. To show the detection threshold in different SERS detections more directly, we summarize the substrates and detection limits in Table 3, including the latest applications in COVID-19 detection [112].

Although it provides a convenient detection method, SERS still faces the following challenges. First, SERS can monitor only live SARS-CoV-2, which has high infectivity. Second, continuous mutation of SARS-CoV-2 strains will increase the complexity of the SERS spectral database and the difficulty

TABLE 3 | Substrates and detection limits in SERS detection.

Detection method	Target/substrates	Threshold/accuracy	References
Label-free SERS in bacterial detection	Ag nanocrystals	Detects as few as 10 colony forming units/mL.	[89]
	Au nanostructure	Concentrations between 10^5 and 10^8 cfu/mL	[90]
	Ag nanorods	100% accuracy in predicting test samples with PLS-DA	[91]
	Au nanorods	100% discriminant rate during classification with LDA	[93]
	Ag NPs	LOD of 10^3 cfu/mL	[94]
	Ag NPs	LOD of 2.5×10^2 cells/mL	[95]
	Ag NPs	No mention; 1×10^6 cells/mL used to detect	[96]
Label SERS in bacterial detection	Single-walled carbon nanotubes/Au NPs	LOD of 1.0×10^2 cfu/mL	[97]
	Au-coated magnetic NPs	LOD of 10 cells/mL	[98]
	AuNPs with specific antibody	LOD of 70 cfu/ mL	[99]
	Aptamer-Fe3O4@Au magnetic NPs	LOD of 3 cells/mL	[100]
	Au@Ag-apt1-target-apt2-X-rhodamine	LOD of 15 cfu/mL	[101]
	Au nanosensor using phages as bioprobes	No mention; concentrations between 10^4 and10^9 cfu/mL	[102]
	Au nanorods using phages as bioprobes	No mention; 10^8 cfu/mL used to detect.	[103]
	Au@Ag NPs modified with 4-MBA	LOD of 13 cfu/mL	[104]
	Au@Ag NPs modified with 4-MBA and graphene oxide	LOD of 1.0×10^3 cfu/mL	[105]
	Ag NPs modified with 4-Mph as probes	No mention	[106]
	Au NPs	LOD of 0.67×10 cfu/mL	[107]
SERS in SARS-CoV-2 detection	Au nanopopcorn using a spike protein deoxyribonucleic acid aptamer as a receptor	LOD of 10 PFU/mL	[109]
	Ag-nanorods functionalized with ACE2	Different locations with different copies/L	[110]
	Au nanostructure functionalized with ACE2	LOD of 80 copies/mL	[111]
	Ag nanocubes.	No mention; 99.9% classification specificity using PLS-DA	[112]

Abbreviations: ACE2 (angiotensin converting enzyme 2), LOD (limit of detection), LDA (linear discriminant analysis), PLS-DA (partial least-squares discriminant analysis).

in using the algorithm. In response to these challenges, SERS is now gradually using pseudo-viruses to simulate all surface proteins of SARS-CoV-2 for detection. Genetic changes among different mutants did not lead to significant differences in spectral peaks—a factor requiring particular attention.

MULTIPLE SPECTROSCOPY TECHNOLOGIES IN PATHOGEN DETECTION

Increasing attention is being paid to the combination of multiple spectroscopy technologies in the detection of pathogenic microorganisms. FTIR and RS have been combined to detect differences in the biochemical compositions of 89 strains of *Listeria monocytogenes*, and the composition of carbohydrates has been found to be the most important characteristic, which may be associated with properties of the cell wall [113]. Furthermore, FTIR and RS have also been combined to quantitatively label and distinguish *Escherichia coli* cells [76]. The novelty of this method involves cultivating *Escherichia coli* cells with different ratios of isotopically labelled ^{13}C glucose and ^{15}N ammonium chloride as the sole carbon and nitrogen sources, respectively [114]. SERS and RRS have been combined to detect growing *Pseudomonas aeruginosa* biofilms, and a characteristic SERS

signal has been identified in the detection of pyocyanin under near-IR (785 nm) laser irradiation [115].

Studies have shown that SERS combined with other techniques can specifically detect and recognize SARS-CoV-2 spike protein [116-118]. Compared with the noble metal NPs, semiconductor materials have higher biocompatibility and spectral stability, thus making them suitable for viral detection [116]. A detection limit of 5×10^{-9} M SARS-CoV-2 protein has been reported. The lateral flow immunoassay (LIFA) is a crucial tool to detect viral infection dynamics. A two-channel SERS-based LIFA biosensor has been used to detect anti-SARS-CoV-2 IgM/IgG [119]. Unlike previously used Au NPs as a SERS reporter, SERS-encoded NPs (SiO_2@Ag NPs) have been innovatively used as ideal SERS tags offering more specific and stable SERS signals. This method can screen COVID-19 rapidly in early infection stages, when the IgM and IgG antibody levels remain low and difficult to detect. Moreover, SERS probes called gap enhanced Raman nanotags have been used to protect Raman reporters from external influences, thus improving SERS signals by 30-fold over those of traditional nanotags [120]. In this study, anti-SARS-CoV-2 IgG and IgM have been detected by SERS-based LIFA, with detection limits of 1 ng/mL and 0.1 ng/mL, respectively. Platforms for SERS coupled with microfluidics has been developed to detect SARS-CoV-2 [117]. The first was a microfluidic device with SERS strips, and the second used vertically aligned carbon nanotubes with Au/Ag NPs. These methods isolate and enrich viruses without tedious steps, such as labeling samples. IRAS combined with RS has been used as a diagnostic technique to detect COVID-19 samples by analysis of the structures of proteins and nucleic acids [118].

ADVANTAGES, DISADVANTAGES AND APPLICATION PROSPECTS OF THE THREE SPECTRAL DETECTION TECHNIQUES

The sensitivity of LIFS is two to three orders of magnitude higher than the other trace analysis methods, such as colorimetry and UV-visible spectrophotometry. LIFS can achieve single molecule detection when combined with other techniques, such as capillary electrophoresis separation [121]. Beyond providing information about the excitation spectrum, emission spectrum, peak position and peak intensity, LIFS can also compensate for the inability of UV-visible absorption spectroscopy to detect chromogenic clusters and their environmental changes [122]. Depending on the differences in pathogen structure, and the excitation and emission wavelengths of fluorescent substances, LIFS can select the appropriate detection wavelength to achieve the selective detection of pathogenic microorganisms. Nevertheless, owing to the small amounts and similar compositions of fluorescent substances in microorganisms, a lack of characteristic peaks in a fluorescence spectrum affects the classification and identification of pathogenic bacteria.

IRAS is non-destructive to samples and is not limited by sample morphology, whereas RS requires only small amounts or areas of sample, because the diameter of the laser beam is usually only 0.2–2 mm at its focus site [123]. In the qualitative and quantitative analysis of pathogenic microorganisms, both IRAS and RS can provide information on functional groups, chemical bonds, and three-dimensional structures, and can complement each other. However, samples dissolved in water cannot be detected by IRAS, because water produces IR absorption and erodes the salt window; in contrast, because the Raman scattering of water is very weak, RS performs better in the detection of water-soluble biological sample [88]. The shortcomings of RS relate to both the overlap of vibrational peaks caused by weak spectral intensity, and the changes of the spectrum morphology, which are affected by the species, growth environments and growth states of the bacteria [124].

Three spectroscopy technologies, whether applied alone or in combination, substantially decrease the workload, financial and material resources, and time required, while enabling the diagnosis, classification and real-time tracking of pathogenic microorganisms with favourable replicability and high resolution. Their application is important for the rapid recognition of pathogenic microorganisms, to enable prevention and control measures, guide the use and screening of drugs or prevent the spread of drug-resistant bacteria. These methods have great application value and prospects, and have been successfully used in various fields such as biologic safety, food safety and pollution and environmental monitoring.

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CONFLICTS OF INTEREST

None.

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