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Advances in detection of fastidious bacteria: From microscopic observation to molecular biosensors

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ABSTRACT

Identification of pathogens and diagnosis of infections are important health challenges, especially in the case of fastidious bacteria which are those difficult-to-grow. A fastidious organism is any organism that has a complex nutritional requirement. Additionally, a fastidious microorganism will only grow when specific nutrients are included in the culture medium. These bacteria can cause serious diseases whose detection and monitoring is critical in many cases. The oldest detection methods are based on simple microscopy observation and staining, after culture on selective growth media, but often do not provide a clear answer. Some new molecular approaches, such as DNA-based sequencing and antibody-antigen binding, are particularly promising in the case of fastidious bacteria. The objective of this review is to summarize the advantages of the use of biosensors as a modern diagnostic tool in microbiology for detecting fastidious bacteria.

1. Introduction

Successful treatment of infectious diseases requires a specific and accurate diagnosis of the disease and thus a fast and selective detection of the presence of bacteria that causes these illnesses. Traditional microbiological methods; such as those based on culturing, staining and microscopy counting and conventional biochemical methods are very difficult to carry out in some cases and highly time-consuming. The use of traditional microbiology detection methods can delay and lengthen the course of treatment [1].

According to public health authorities, infectious diseases are still one of the most important causes of human mortality in the world, especially in less-developed nations. The control and monitoring of infectious diseases requires improvement in microbiological diagnostic methods. For a long time, bacterial culture in appropriate laboratory culture media has been the gold standard for pathogen detection [2]. However, it must be taken into consideration that often only a single bacterial species out of thousands inhabiting the human body, or out hundreds found in food or water can cause an infectious disease. So, rapid diagnosis with high sensitivity and specificity for the presence of pathogens is crucial for disease diagnosis. The most important limitation of traditional methods is the fact that they are very time-consuming.

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Additional limitations are their relatively low sensitivity and specificity. Moreover, traditional microbiological methods may be unsafe for operators and require costly and sophisticated equipment not usually available in less-developed regions of the world. In response to these limitations, molecular methods have emerged, providing an advance in diagnostic methods. These methods have partly resolved the limitations of the old methods, although even these methods are still not ideal [3].

One of the newest methods for detecting pathogens, especially difficult-to-culture pathogens, is the development of biosensors and, in this field, the use of nanomaterials together with biological systems has expanded biosensor applications to be a powerful tool for detection of difficult bacteria. In the following sections, the detection of some bacteria, such as: *Legionella pneumophila, Brucella, Francisella tularensis, Borrelia burgdorferi, Leptospiraceae* family, *Mycobacterium tuberculosis, Hemophilus influenza, Helicobacter pylori, Mycoplasma, Campylobacter jejuni* and *Treponema pallidum,* will be considered as clear examples of the advantages of moving from classical methods to the use of biosensors in order to overcome many of the limitations of traditional methods [4].

2. Fastidious bacteria

The most important feature of fastidious bacteria is that they have a very complex set of nutritional requirements. So, these bacteria do not grow at all in simple laboratory culture media, that would be suitable for most regular bacteria. Additionally, the doubling time of these bacteria colonies is very slow, and in some cases can involve days or even weeks.

In order to provide to the reader a description of the group of bacteria which will be evaluated in this review, Table 1 summarizes the main characteristics of some fastidious microorganisms together with data about the associated infections, transmission routes and main symptoms in humans.

From data summarized in Table 1 it can be concluded that there is tremendous interest in robust and highly sensitive and selective methods for the detection of fastidious bacteria and the diagnosis of the related infectious diseases.

3. Traditional methods used for the detection of fastidious bacteria

Traditionally, detection of bacteria has been based on direct observation or measurements made after bacterial growth in an appropriate culture medium.

Anthony Leeuwenhoek was the first to observe the structure of bacteria in 1676. He used a very simple microscope to do this, and first published his observations in London. Nowadays, there are a wide variety of microscope types that can be used to directly observe bacteria. These include: ordinary (light) microscopy (bright-field and dark-field), phase contrast, differential contrast, fluorescence, laser scanning, transmission, scanning and tunneling electron microscopy, and many other forms of microscopy. The most important limitation in microscopic observation is that very small pathogens, such as viruses and some bacteria, are not easily visible with light microscopy. Additionally, since transparent objects, such as bacteria, were not visible using the early microscopes, different staining methods were developed and thus, a wide variety of stains have been proposed, particularly for bacteria. The most important laboratory procedures being the Gram stain and the acid-fast method. Advanced staining methods, such as fluorescence staining, provide the ability to view both internal and external structures of bacteria [15]. Gram staining dates from very early times, but is still widely used today. According to this method, based on the structure of the cell-wall, Gram-positive bacteria are stained a purplish color while Gram-negative bacteria assume a pinkish color. The general morphological appearance and shape along with gram staining can identify a wide range of bacteria. When used on acid-fast bacteria, Gram staining is not effective and the special stains and other methods should be used [16].

In summary, the following points relate to the microscopic observation method: i) despite the simplicity of the method, it could be very specific; ii) microscopic observation is a very old but reliable method; iii) the direct observation method is almost always cost-effective; iv) given the direct exposure to bacteria, this method may not always be safe, with some risk of infection; v) the direct microscopy observation method is not a universal method because it cannot be carried out with all types of bacteria. Despite significant advances in pathogen identification, culture is still a gold standard for the detection of some bacteria. According to Koch's postulates, pure microbiological culture is the basis of all research on infectious disease. The first isolation of bacteria not only led to the emergence of a new model for the analysis of pathogenicity of infections, and the formulation of the Koch's postulates, but also led to establishing the link between the presence of microorganisms and the incidence of infectious disease. Primary culture media were established on the basis of naturally occurring materials and biological compounds. In general, the four most important determinants of bacterial growth are: temperature, atmosphere, nutrition, and incubation time [17]. Despite the existence of some limitations, the identification of bacteria on the basis of cultivation on media is a reliable method. One of the characteristics of bacterial culture is that the number of individual bacteria increases exponentially, so that single bacterial cells eventually become colonies of pure bacterial species. The most important limitation to the culture method is it is time-consuming and, in some cases, the incubation time can be much longer than one day, and in some cases, one or more weeks are needed to ensure the appropriate growth of colonies. The above-mentioned problems are particularly troublesome in industrial settings, particularly in the food industry. However, culture based methods are still fundamental to microbiology and further advancements can still be made [18].

In the particular case of fastidious bacteria, different laboratory culture media have been proposed and Table 2, summarizes some of the compositions found in the scientific literature. Table 2 also indicates the main problems with the growth of fastidious bacteria.

From data summarized in Table 2, the following points should be borne in mind when culturing fastidious bacteria: i) culture techniques can be difficult and time consuming; ii) despite the limitations and difficulty, culture is still the gold standard for detection of many bacteria; iii) some fastidious bacteria, such as *M. tuberculosis* and *F. tularensis*, are very hazardous, and thus cultivation requires professional equipment and personnel and is only possible in reference laboratories; iv) few fastidious bacteria are obligatory intracellular and their cultivation requires different treatments and handling; v) some difficult bacteria are in a VBNC (viable but not cultivatable) state, in which case, culture is not possible; and vi) few fastidious bacterial species are totally uncultivatable, such as *Mycobacterium leprae*.

Serologic studies include the use of serum and other body fluids for bacterial detection in without the need for laboratory cultivation. The term serology refers to the examination of antibodies against bacteria present in the serum. Antibodies are proteins that are secreted by B-lymphocytes, after recognition of a pathogen, an external protein or antigen, or in some circumstances against host proteins themselves. There are several different types of serologic testing that can be carried out with a wide range of different bacteria. ELISA, agglutination, precipitation, complement-fixation, fluorescent antibodies, and more recently chemiluminescence are the main

Table 1
Characteristics of studied fastidious bacteria and their associated infections.

Bacteria	Description	Infection	Transmission	Symptoms	Ref
Legionella	Pathogenic group of Gram-negative bacteria that includes the species *L. pneumophila*.	Legionnaires disease/ Pontiac disease	Airborne transmission from cooling towers, Person-to-person transmission has not been demonstrated	Prodromal symptoms are flu-like, including fever, chills, and dry cough, Advanced stages of the disease cause problems with the gastrointestinal tract and the nervous system and lead to diarrhea and nausea.	[5]
Brucella	*Brucella* is a genus of Gram-negative bacteria, They are small, non-encapsulated, non motile, facultative intracellular coccobacilli	Brucellosis, Malta fever/ Bang's disease, Undulant fever/ enzootic abortion.	Transmitted by ingesting contaminated food (such as unpasteurized milk products), direct contact with an infected animal, or inhalation of aerosols.	Gastric fever/ram epididymitis, abdominal pain, vomiting, diarrhea, constipation, hepatomegaly, and splenomegaly	[6]
Francisella tularensis	Gram-negative coccobacillus, an aerobic bacterium, causative agent of tularemia, the pneumonic form of which is often lethal without treatment	Tularemia, pneumonic form of which is often lethal without treatment	Ease of spread by aerosol, not transmitted by human to human	Oropharyngeal infection, ulcer glandular	[7]
Borrelia burgdorferi	*Borrelia burgdorferi* is a bacterial species of the spirochete class of the genus *Borrelia*.	Lyme disease	Erythema migrants (EM), uniformly erythematous, Arthritis, Facial nerve palsy, Meningitis, Endocarditis	Hard-bodied ticks, Nymphal ticks	[8]
Leptospira	Corkscrew-shaped bacteria	Leptospirosis, Weil's disease, severe pulmonary hemorrhage syndrome	Fever accompanied by chills, intense headache, severe myalgia (muscle ache), abdominal pain	Transmitted by both wild and domestic animals	[9]
Mycobacteria	Mycobacteria are aerobic, They are bacillary in form, Acid fast bacteria that is a genus of Actinobacteria,	Tuberculosis, leprosy (*Mycobacterium leprae*)	Cough, fever, and/or weight loss	Persons who have TB disease are usually infectious and may spread the bacteria to other people.	[10]
Haemophilus influenza	Gram-negative, coccobacillary, facultatively anaerobic pathogenic bacterium belonging to the Pasteurellaceae family	Meningitis, bacteremia, pneumonia, epiglottitis, cellulitis, osteomyelitis, and infectious arthritis.	Cough Shortness of breath Chills Sweating Chest pain that comes and goes with breathing Headache Muscle pain	Aerosol, person to person	[11]
Helicobacter pylori	Gram-negative, microaerophilic bacterium usually found in the stomach.	Gastritis, duodenal ulcers and stomach cancer, gastric ulcers	Nonulcer dyspepsia: stomach pains, nausea, bloating, belching, and sometimes vomiting or black stool.	Exact route of transmission is not known, Person-to-person transmission by either the oral–oral or fecal–oral route is most likely.	[12]
Mycoplasma/ Ureaplasma	Genus of bacteria that lack a cell wall around their cell membranes	Infertility, atypical pneumonia, pelvic inflammatory diseases	Cells gradually shift from their normal form to sickle-shaped, hyperchromatic cells	Sexually transmitted infections	[13]
Campylobacter jejuni	Among the most common bacterial infections of humans, often a foodborne illness.	Gastritis, diarrhea or dysentery syndrome, mostly including cramps, fever	The prodromal symptoms are fever, headache, and myalgia.	The common routes of transmission for the disease-causing bacteria are fecal-oral, person-to-person sexual contact, ingestion of contaminated food (generally unpasteurized (raw) milk and undercooked or poorly handled poultry), and waterborne (i.e., through contaminated drinking water).	[14]

serological techniques used for bacterial detection. Serologic tests in some cases are not limited to blood samples, but can be applied to other bodily fluids such as CSF, semen and even saliva.

The following points should be borne in mind for serological detection of fastidious bacteria: i) In many cases, serology tests can display false positive and false negative results, therefore, they are not completely reliable for infection diagnostic; ii) in some cases, such as *brucella* detection, serological testing is labor-intensive and is usually used as a confirmatory test despite that it is labor-intensive; iii) serology-based methods have in general, lower sensitivity than culture methods; and iv) generally an alternative method to serological testing should be used to confirm the accuracy of results.

Nowadays molecular based detection methods are paramount in detection of fastidious microorganisms. The discovery of nucleic acids was one of the most significant and noteworthy developments associated with infectious disease. Nucleic acid identification of a microorganism is commonly carried out using the PCR technique, especially for uncultivatable and fastidious bacteria. Nowadays, molecular tests including: PCR, DNA microarrays, and nucleic acid sequencing, have play an important role in the clinical laboratory. Molecular tests have high sensitivity and specificity compared to culture and serology methods. Recent progress in high-density or highly parallel sequencing techniques has removed the limitation of requiring an a priori target selection, which was inherent to traditional PCR/probe-based assays. Molecular diagnostics have the capability to decrease the time to produce results and can provide a high precise diagnosis. Despite these clear advantages, molecular diagnostic methods are not free from problems [20].

Table 2
Culture media studied for detection of fastidious bacteria.

Bacteria	Media	Problems	Ref
Legionella	Buffered yeast extract (BYE) broth, buffered charcoal yeast extract (BCYE) agar	Fastidious, hard to grow	[5]
Brucella	Brucella agar, modified Thayer-Martin medium	Prolonged incubation (up to six weeks) may be required	[6]
Francisella tularensis	Chocolate agar and Thayer-Martin medium	Growth is slow, fastidious	[7]
Borrelia burgdorferi	Barbour-Stoenner-Kelly media (BSK media)	Fastidious, hard to grow,	[19]
Leptospira	Ellinghausen, McCullough, Johnson, and Harris (EMJH) medium.	Fastidious, hard to grow, varied sensitivity	[9]
Mycobacteria	Lowenstein–Jensen medium, Loffler medium, Middlebrooks 7H11 agar	Fastidious, hard to grow, unsafe	[10]
Haemophilus influenza	Chocolate agar, with added X (hemin) and V (nicotinamide adenine dinucleotide)	Fastidious, hard to grow	[11]
Helicobacter pylori	Supplement agar with cyclodextrin B	Fastidious, hard to grow	[12]
Mycoplasma/Urea plasma	Culture tests are rarely used	Not recommended	[13]
Campylobacter jejuni	Selective blood agar medium (Skirrow's medium), actidione [Preston's agar]	Fastidious, hard to grow	[14]

The following points should be borne in mind for detection of fastidious bacteria using molecular methods: i) molecular methods have an increased sensitivity as compared to culture and especially serological methods; ii) these methods are much faster than culture ones; iii) for uncultivatable bacteria and VBNC, molecular methods are the ideal choice; iv) molecular approaches are relatively expensive compared to culture and serological tests; v) performing molecular tests and interpreting their results require qualified personnel and also dedicated laboratory equipment.

As a summary of the different points considered in the preceding sections, it can be concluded that the traditional methods employed for fastidious bacteria detection are, in general, time consuming and lacking in sensitivity. The new methods based on PCR are more expensive alternatives for the diagnosis of associated infections, which may only be justified for high severity diseases. Table 3 summarizes the aforementioned aspects of the use of traditional methods.

4. Biosensors

Biosensors are powerful tools that can produce information on nearly any chemical, physiological, or biochemical analyte or biological process. A biosensor is a probe that integrates a biological analyte recognition system with an electronic transducer thus converting a biochemical signal into a measurable electrical or optical signal. Biosensors have relatively simple structures composed of: (a) a bio-receptor or biological recognition component; (b) a signal transducer; and (c) an amplifier. The interaction between the analyte and the bio-receptor is transformed into quantifiable signals. Various bio-receptors for example: enzymes, antigens, nucleic acids, antibodies, tissues and whole cells can be used in biosensor design. A variety of transducers such as electrochemical, optical, acoustic and electronic can be used for these devices [31].

Biosensors combine the specificity and sensitivity of biological recognition systems with the power of transducers in order to carry out simple, easy-to-use bioanalytical measurements. They have applications in a wide range of fields such as diagnosis, drug control, environmental monitoring and food safety. Electroanalytical methods are the most common sensing approaches due to their innate high sensitivity and ease of use that can be successfully carried out with miniaturized hardware. The most common types of electrical biosensors that have been applied to infectious disease diagnosis include voltammetry, amperometric, impedance and potentiometric sensors [32].

Some properties are important in biosensor fabrication for sensing the presence of microorganisms. The biosensor must provide suitable values of: accuracy, assay time, sensitivity, specificity, reproducibility, robustness, ease of use and a compatible interface. So, sensors must have no false negative results, and false positive results must be very rare, it is important that they could provide as near real-time response as is possible, where less than one-hour is desirable, when the infectious dose required for detection is between 1 and 1000 cells, it must aline with the sensitivity level of biosensors. The specificity of the device should allow it to distinguish between the target and the other similar species. Biosensors for bacterial detection must distinguish different types of

Table 3
Conventional methods for the detection of fastidious bacteria.

Bacteria	Disease	Disease severity/mortality	Routine detection tests	Comments	Ref
Legionella	Legionnaires disease/Pontiac disease	Low	Culture/PCR	Time consuming/unreliable	[21]
Brucella	Brucellosis	High	Culture/Serology/PCR	Due to its ease of spread by aerosol and its high virulence, culture is not recommended	[22]
Francisella tularensis	Tularemia	High	Culture/Molecular based tests	Due to its low infectious dose, ease of spread by aerosol, and high virulence, culture is not recommended	[23]
Borrelia burgdorferi	Lyme disease	Low	Serology/Real Time PCR	Time consuming/expensive	[24]
Leptospira	Leptospirosis	Low	Serology/Culture	Time consuming/false negative and positive result	[25]
Mycobacteria	Tuberculosis	High	Culture/PCR	Time consuming and high pathogenicity/expensive	[26]
Haemophilus influenza	Meningitis	Low	Culture/PCR	Time consuming/expensive	[27]
Helicobacter pylori	Gastritis	Low	Culture/Serology/PCR	Time consuming/expensive	[28]
Mycoplasma/Ureaplasma	Infertility	Low	Serology/PCR	Low sensitivity	[29]
Campylobacter jejuni	Gastritis	Low	Culture/PCR	Time consuming/expensive	[30]

microorganisms and must provide a reproducibility of around 90%. Both, mechanical and chemical stability, are required and devices should be easy to be automated and need minimal operator skills, being well-matched with the transduction principle and avoid non-specific binding. Additionally, biosensor performance should be compared against current standard techniques and be able to obtain an appropriate limit of detection (LOD) [33].

4.1. Surface plasmon resonance (SPR)

Surface Plasmon Resonance (SPR) can be employed in genosensors and immunosensors for fastidious bacteria detection, offering alternative methods for the diagnosis of their associated infections. Surface plasmon resonance is based on the excitation of an electromagnetic wave spreading along the interface of two media with dielectric constants of opposite signs (such as metal and sample buffer) using a light beam with a specific angle of incidence. The signal obtained is based on total internal reflection that results in the reduced intensity of the reflected light. The angle at which the resonance occurs is sensitive to any change at the interface such as changes in refractive index or formation of a film with nanoscale thickness due to surface molecular interactions. Therefore, these changes can be measured by monitoring the shift of the light intensity minimum over time [34]. The schematic configuration of an SPR detector is illustrated in Fig. 1.

A SPR based biosensor was designed in 2017 for detection of *Legionella* in the form of a scalable sensing prototype for the direct detection of this bacterial species in water and air samples of working environments, providing a method that could be used by non-specialized personnel [36]. Another study reported the detection of protein G in *Legionella* by monitoring the surface morphology of the self-assembled protein G layer on an Au substrate and a monoclonal antibody against *L. pneumophila* using atomic force microscope (AFM) [37].

In 2015, SPR based sensors were used to identify *Neisseria meningitides*. A highly oriented ZnO thin film of thickness 200 nm was deposited on gold coated glass prisms by the RF sputtering technique. The SPR biosensor provided high specificity and long lifetime [38].

The following points are relevant for SPR-based detection methods: i) provides accurate results, as replicates consistently show the same results; ii) can be used to test crude samples in applications such as serum analysis; iii) some SPR biosensors can be reused, lowering the cost of consumables; iv) requires reduced amounts of sample for binding kinetic experiments.

4.2. Genosensors

The electrochemical DNA biosensor is a novel biosensor that was developed in recent years. Detection of microbial DNA sequences is particularly important for clinical diagnosis and food safety. Using DNA sequencing analysis, we can identify the cause of the disease even if there are no clinical symptoms. Detection of specific DNA sequences can be used for pathogenic bacteria, fungi or virus detection. DNA biosensors and gene chips have a remarkable ability to provide sequence-specific information in a simple,

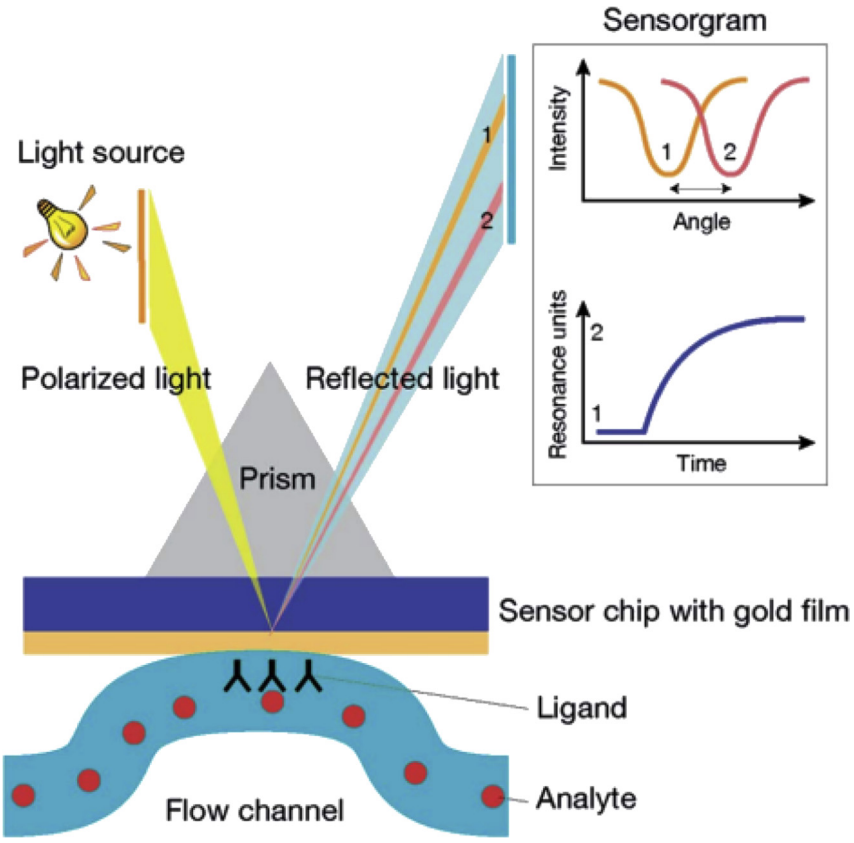

Fig. 1. Schematic configuration of an SPR detector. The incident polarized light is coupled by a glass prism on the biosensor chip coated with a thin layer of gold and integrated with a flow channel for continuous flow of buffer. At a defined incidence angle, the SPR phenomenon is seen as a dip in the intensity of the reflected light, characteristic of the specific angle of reflection. The shift of the angle of reflection from position 1 to position 2 reveals a change in the composition of the medium near the gold film as a result of the binding between the ligand and the analyte. The angular variations are recorded in resonance units (RU) and plotted versus time in a sensorgram [35].

rapid and low-cost manner, compared to traditional hybridization techniques. DNA biosensors, based on nucleic acid recognition, provide rapid, economical and simple assays for genetic and infectious diseases. Generally DNA sensors contain a single-stranded DNA sequence immobilized on a particular substrate As soon as the complementary DNA sequence binds to the DNA probe, an electrical current is generated, which is recorded by the device read-out mechanism. In many DNA biosensors, the probes are directly immobilized on the electrode. The DNA fragments have to be immobilized in such a way as to maintain their stability and orientation, in order to preserve reactivity and ease of access to the target analyte [39]. Simplicity, small size, and convenient assays that can be easily operated even by non-professional users, are the advantages of electrochemical genosensors. Genosensors offer the possibility to be miniaturized and are usually low cost, offering high sensitivity and specificity. A wide variety of biosensors have been developed to detect fastidious bacteria.

A genosensor was evaluated for the assay of *Brucella* DNA in culture media and human samples with and without PCR. The genosensor could detect the complementary sequence with a sensitivity of 0.02 μA dm^3 mol^{-1}. Palladium nanoparticles were potentiostatically electrodeposited onto a gold surface at a highly negative potential [40]. Additionally, an oligonucleotide probe was designed for the optical detection of *Brucella* spp in a study by Sattarahmady et al. [41]. Gold nanoparticles (AuNPs) were functionalized with a specific oligonucleotide probe designed to recognize the IS711 gene region. After attaching the probe to the AuNPs, the biosensor was mixed with target and non-target oligonucleotides, and *Brucella* genomic DNA extracted from clinical samples. Results were measured by UV-VIS spectrophotometry, relying on the color change exhibited by AuNPs when they aggregate. The results were also visible to the naked eye. In acidic conditions the tubes containing target complementary DNA were red, while those with non-target DNA were a blue-purple color. Using this method, 10.09 pg L^{-1} of genomic DNA could be detected. Given that *Brucella* is a facultative intracellular bacterium, the DNA could also be amplified by PCR before the colorimetric assay. High sensitivity and selectivity were shown [41].

In 2015, Rahiet et al. used a nanostructure to immobilize a *Brucella*-specific probe creating a genosensor with a transducer, and used an electrochemical system to detect hybridization. Methylene blue was also used as a redox reporter. They could measure both non-complementary, complementary and base-mismatched sequences to detect bacteria in culture media and samples taken from patients without PCR amplification. They reported a sensitivity of 0.40 μA, a linear concentration range between 10 zmol/L and 10 pmol/L, and a detection limit of 1.71 zmol/L [42].

Detection of *Mycoplasma. pneumonia* is one of the great challenges of diagnostic in microbiology. 3,4,9,10-Perylenetetracarboxylic acid anhydride (PTCDA; a π-stacking perylene semiconductor with high intrinsic optical absorption) was coated onto a gold electrode and a layer of gold nanoparticles (nano-Au) was used to immobilize DNA. This diagnostic method showed advantages of low cost, high performance and high sensitivity and specificity (See Fig. 2 for details) [43].

Recently, Chen et al. described a powerful biosensor to detect *M. ovipneumoniae* (MO, the causative agent of pneumonia in sheep) based on manganese dioxide (MnO_2) microspheres attached to a Cy5-labeled single stranded DNA (Cy5-ssDNA) probe complementary to the MO conserved DNA sequence. The fluorescence enhancement showed a good linear relationship with the concentration of a MO plasmid with a detection limit of 1.042 copies/L and a dynamic range of 10^2–10^6 copies/L. Good reproducibility and stability were achieved, and the sensor could distinguish between a perfect complementary target match and a single base-mismatched DNA sequence. In terms of physicochemical properties, microspheres were better than Nano sheets for interaction with the Cy5-ssDNA probe [44].

In 2010, Sun et al. designed a DNA based biosensor for the detection of *Yersinia. enterocolitica*. In this study, vanadium pentoxide nano-belts (nano-V_2O_5), multi-walled carbon nanotubes (MWCNTs) and chitosan (CTS) were used to prepare a nanocomposite material with a carbon ionic liquid electrode (CILE) as the working electrode. Methylene blue (MB) was used as indicator to screen the hybridization response with the target ssDNA sequence. The amounts of ssDNA adsorbed on the electrode surface was increased due to the synergistic effect of nano-V_2O_5 and MWCNTs, improving the electrochemical response. The DNA biosensor discriminated between the one-base and three-base mismatched ssDNA sequences [45].

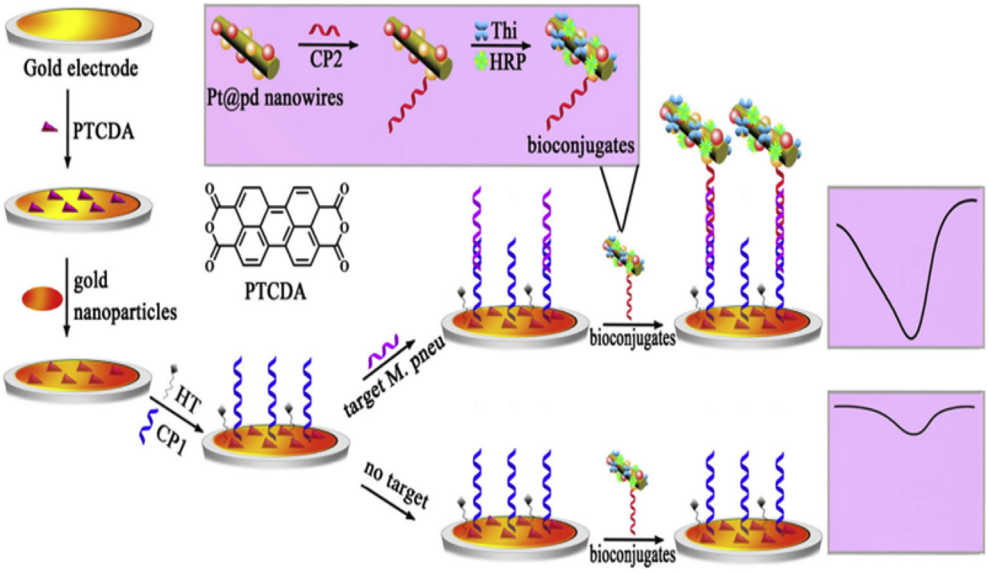

Fig. 2. The assembly of an electrochemical DNA biosensor for *Mycoplasma. Pneumonia* detection (PTCDA: 3,4,9,10- perylenetetracarboxylic anhydride, HT: hexane thiol, CP1: capture probe, CP2: complementary probe, HRP: horseradish peroxidase, Thi: thionine) [43].

A sensing platform, using reduced graphene oxide-gold nanoparticles (rGO-AuNPs) as the DNA probe immobilization substrate and AuNPs-polyaniline (Au-PANI) as the reporter label, was reported for the highly sensitive recognition of the specific *Mycobacterium tuberculosis* DNA insertion sequence. A large surface area was obtained due to the use of reduced graphene oxide. This platform had good stability and high specificity for clinical diagnosis of *M. tuberculosis* [46].

Torres-Chavolla and co-workers designed a biosensor to detect *M. tuberculosis*. In this study, thermophilic helicase-dependent isothermal amplification (tHDA) was used, combined with dextrin-coated AuNPs as an electrochemical reporter for detection of target DNA. The AuNPs and amine-terminated magnetic particles (MPs) were each functionalized by a different DNA probe that precisely hybridized with opposite ends of a fragment within the IS6110 gene sequence, specific for *M. tuberculosis* complex (MTC). AuNPs were electrochemically deposited on a screen-printed carbon electrode (SPCE) chip. After hybridization, the complex formed (MP-target-AuNP) was magnetically separated from the solution. The detection limit was 0.01 ng/L of isothermally amplified target (105 bp) [47].

Drug resistance is a major challenge in the war against tuberculosis. Identifying genes involved in drug resistance is very important in optimization of drug therapy. Oligonucleotide sequences related to the normal and mutated rpoB genes of *M. tuberculosis* were detected using a surface plasmon resonance (SPR) biosensor system. Oligonucleotides matching the sequence contains the mutated TCG: TTG codon of the rpoB gene of *M. tuberculosis* were thiol-modified to be attached onto a gold sensor surface. After specific hybridization between immobilized probe P2 and complementary target T2, the maximum sensor response was obtained. In the case of a single-base mismatched oligonucleotide (TN) a lower response, and for an irrelevant oligonucleotide (TC), no response was observed [48].

Neisseria gonorrhoeae is responsible for a major sexually transmitted disease (STD), called gonorrhea. An electrochemical genosensor was designed using a thiolated probe recognizing the *opa* gene of *N. gonorrhoeae*. 6-Mercapto-1-hexanol (MCH) was used as a blocking agent to allow the oligoes to "stand" up at the surface. The biosensor had a wide dynamic range (from 1.0 × 10-6 M to 0.5 × 10−18 M) and a low detection limit (1.0 × 10−18 M). Stable hybridization was detected using methylene blue as an electroactive DNA hybridization indicator [49].

Tak et al., used zinc oxide (ZnO) nanostructures with a particular morphology incorporated on a platinized silicon substrate, together with cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) to monitor the binding of the ss th-DNA probe against *N. meningitides* on the nanostructured ZnO (ZNF) matrix surface. They also tested poly-(4-amino-3-hydroxynapthalene sulfonic acid) (*p*-AHNSA) modified pyrolytic graphite edge plane. The electrochemical sensing performance gave a low detection limit of about 5 ng μL^{-1}, good linearity (R¼ 0.98) in the range 5–240 ng μL^{-1}, and high sensitivity (168.64 $\mu A\ ng^{-1}\ \mu l\ cm^2$) [50].

In general, in conjunction with *Legionella* bacteria, there are fewer biosners compared to other bacteria. An electrochemical genosensor was described for the detection of nucleic acid sequences specific to *L. pneumophila,*. In this platform, biotin was used as a label and correspondingly streptavidin-alkaline phosphatase as the reporter molecule. An immobilized thiolated hairpin probe was combined with a sandwich-type hybridization assay. The activity of the immobilized enzyme was assayed voltammetrically by measuring the quantity of 1-naphthol created in less than 3 min of enzymatic dephosphorylation of 1-naphthyl phosphate. The biosensor had the ability to differentiate the strains of the *Legionella* bacteria and also had high sensitivity and specificity. A linear association between the analytical signal and the logarithm of the target concentration up to 2 μM was obtained and the limit of detection was 340 pM *L. pneumophila* DNA. The hairpin-based system evaluated was better than similar sandwich-type assays using linear capture probes [51]. In 2015, Foudeh et al. described a biosensor for detection of *L. pneumophila*. One of the most important features of this biosensor was the ability to detect bacteria in

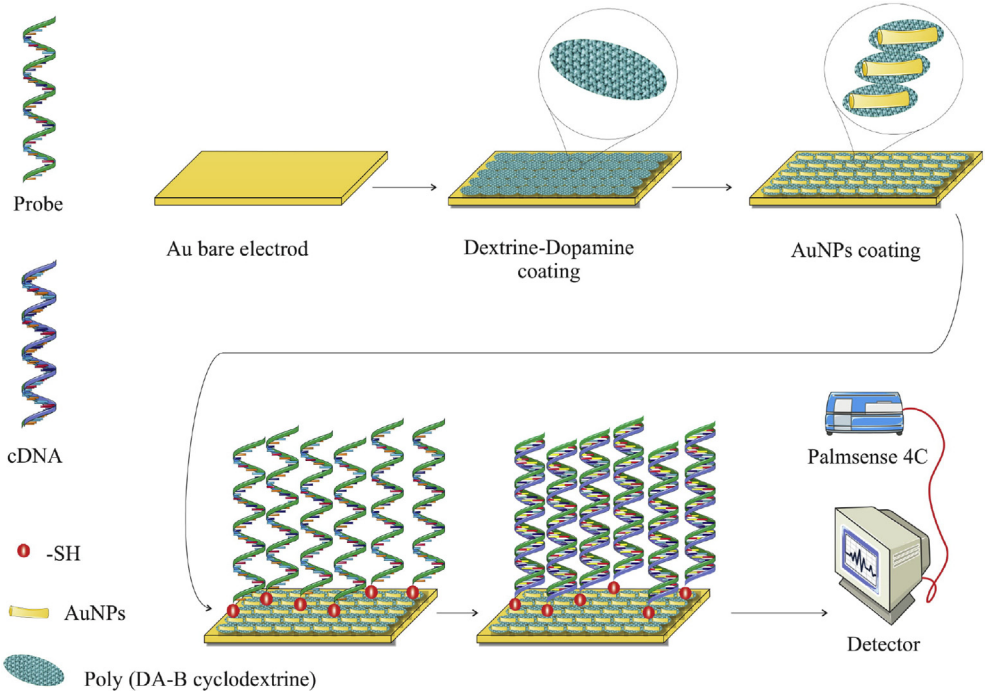

Fig. 3. Schematic illustration of the DNA based biosensor for detection of *L. pneumophila* [52].

Table 4
Recently reported genosensors for fastidious bacteria.

Bacteria	Transducer	Sensitivity	LOD	Gen	Linear Range	Primer sequences	Year-Ref
Brucella	nPd	0.02 mA dm3 mol1	2.7 1020 mol dm3	t-ssDNA	1.0 1012 to 1.0 1019 mol dm3,	Forward primer: 50-GACGAACGGAATTTTTCCAATCCC-30 Reverse primer: 50-TGCCGATCACTTAAGGGCCTTCAT-30	2016-[40]
Neisseria meningitidis	carbon/ODT-cMWCNT	31.825 (μA/cm2)/nM	~81 pM ~68 pM	Omp85	0–5.40 × 102 pM	forward 5′-CGTTTCCCAAGCAACCTGT-3′) reverse primers 5′-AAATTCACGCCGTCGTATTC-3	2014-[40]
Meningitis	gold (Au) coated glass electrode	115.8 lA/ng	7–42 ng/ll	CtrA	7–42 ng/ll	forward 5′-GATACGAATGTGCAGCTGACACG-30 reverse primers 530-CTATGCTTACACGTCGACTGTGC-50	2009-[58]
Meningit is	gold (Au) coated glass electrode	0.0115 A/ng cm-2	10–60 ng/l	ctrA	0.0115 A/ng cm-2 and 0.0056 A/ng cm-2	forward: 5-TGC GGT GGC TGC GGT AGG GT-3 reverse: 5-CGG CGA GAA CAC AAA CGA CAA GA-3	2010-[59]
Mycoplasma pneumoniae	PTCDA	93.33%	0.03 pM	MPN128	0.1 pM to 20 nM	forward primer 5′-TGA ATA GCC AAG TAC ACC G-3′ backward primer 5′-AAC AAT CGC CAT CAG GAC-3′	2016-[43]
Brucella	AuNP	3.17 OD L ng-1	1.36 pg L-1	IS711gen	12.3 ng	forward 5 CTG GCT GAT ACG CCG GAC TTT GAA 3 reverse 5 GGA ACG TGT TGG ATT GAC CTT GAT 3	2015-[41]
N. meningitidis	SPGE	03 ng	9.5087 (μA/cm2)/ng	rmpM	0–12 ng/6 μL	5′-thiol-labeled 19-mer DNA probe (5′-HS-C6H10-GCTCGCT TCCGGCACTGCT-3′)	2013-[60]
M. tuberculosis	rGO–AuNPs	–	–	IS6110	1.0 1015 and 1.0109 M	forward 50-AGAAGGCGTACTCGA-CCTGA-30 reverse 50-GATCGTCTCGGCTAGT-GCAT-30	2014-[46]
M. tuberculosis	SPCE	0.01 ng	–		–	forward: 5 GAG CGT AGG CGT CGG TGA CAA AGG 3 reverse: 5 GCT TCG GAC CAC CAG CAC CTA ACC 3	2011-[47]
N. gonorrhoeae	ssDNA–Au	1.0 × 10–18 M	–			Probe DNA: 5-SH-CCGGTGCTTCATCACCTTAG-3 Complementary target DNA: 5-CTAAGGTGATGAAGCACCGG-3	2010-[61]
Y. enterocolitica	MWCNTs	1.76 × 10–12 mol L-1	2.0 × 10-5 mol L-1			probe ssDNA: 5-CCGGCAAAACGTCTGCGTGA-3, target ssDNA: 5-TCACGCAGACGTTTTGCCGG-3,	2010-[45]
Legionella sp	Au	2.3 × 10–14 M	10-13 to10-6 M	10-14 to 10-6 M	fthA gene	3-HS-(CH2)3GCAACT TGT TTT CCC CGC CCCTCTCATAGTT (CH2)6NH2-5), non-thiolated complementary target sequence (5-ACA AAA GGG GCG GGG AGA GTA-3)	Varun Rai-2012
Legionella pneumophila	AuNPs	20 pM	0.176 A/pM	50–750 pM	Mip	5-AGT-GAA-TTT-TGC-AGA-GAT-GCA-TTA-GTGCCT-TCG-GGA-ACA-CTG-AT-3 Probe: SH–(CH2)3/biotin – 5-ATC-AGT-GTT-CCC-GAA-GGC-ACT-AAT-GCATCT-CTG-CAA-AAT-TCA-CT-3	2010-[62]
Aeromonas hydrophila	gold oxide	11.5 × 107 molecules cm-2	–			forward: 5CTGCGAGGGTTATCGTTGTG backward: 5 GTGTCGCTGTCGTTGATCG	2010-[63]
Legionella pneumophila	MWCNT	100 nM	Within 10 pM to 100 nM	10 pM to 100 nM	Mip	Probe sequence: 5-NH2–TAG CTA CAG ACA AGG ATA AGT-3 Complementary sequence (target): 5-ACT TAT CCT TGT CTG TAG CTA-3	2010-[64]
Legionella pneumo phila				20–100 nM	16srRNA	Forward AAGGGTGCGTAGGTGGTTG Reverse CCTCTCCCATACTCGAGTC	2009-[65]
T. pallidum	AuNPs	0.5 pM	–			Forward: -5TGCGCGTGTGCGAATGGTGTGGTC-3 Reverse: 5 -TGCACATGTACACTGAGTTGACTCGG-3	2010-[66]
Campylo bacter jejuni	AuNPs	2.5 nM	~9.7 × 10–7 RU	(hipO)	2.5 nM DNA	forward (5 - GAC TTC GTG CAG ATA TGG ATG CTT-3) reverse (5 -CGA TAT TGA TAG GCT TCT TCG GTA GTA-3)	2010-[67]
Helicobacter pylori	AuE	0.15 nM,	–	MWG Operon	0.3–240 nM	5′-HS (CH2)6 AGA CAT GCA AAA AGG TAT-3′. Mismatched DNA (*H. pylori*): 5′-AGA CAT GCT AAA AGG TAT-3	2017-[68]
Helicobacter pylori	Au	0.06 μg/m	11.81 × 10–6 A	glmM	0.72–7.92 μg/mL	Forward: 5′-AAGCTTTTAGGGGTGTTAGGGGTT Reverse: 5′-AAGCTTACTTTCTAACACTAACGC-3′T-3′	2011-[69]
Leptospira	AuPNs	3.95 101 genomic equivalent per reaction	8.0101 e3.9104	LipL32 gene	_	CCGACATTCTTTCTACACGGATCGAATTCCCC CAGAAGAAAAATCAATGC 50	2016-[70]
Neisseria meningitidis	SPR	0.03°/(ng/μl)	5 ng/μl	Probe DNA	10–180 ng/μl	5′-HS-GATACGAATGTGCAGCTGACACG-3	2015-[38]
Mycobacterium avium subsp	lateral flow biosensor assay	101 to 106 organisms	0.1 nM	IS*900* sequences	1-1000 fmol μ/l	Forward: 5ACCGTGCGCCCGGGAATATA3 Revers: 5GGAGTTGATTGCGGCGGTGA3	2009-[71]

peripheral blood samples that also contained a protozoan species. To verify the specificity and sensitivity of the detection system, total RNA extracted from *L. pneumophila,* in spiked water, was co-cultured with amoebae. The results revealed that the expression level of ribosomal RNA (rRNA) was dependent on the environmental conditions. The amount of *L. pneumophila* and the expression of 16s rRNA increased due to the presence of amoebae mixed with bacteria. *L. pneumophila* was detected by an advanced surface plasmon resonance imaging (SPRi) detection method [52]. Recently DNA based biosensor fabricated for detection of *L. pneumophila*. In this study AuNPs were used for increased sensitivity of the biosensor. The findings showed good linear range and high selectivity. Fig. 3 [53]. Other genosensors, designed for a range of fastidious bacteria are summarized in Table 4.

In 2005, del Pozo and coworkers fabricated a DNA based electrochemical biosensor for detection of *H. pylori.* They immobilized a thiolated DNA probe on an Au electrode and electrochemical detection was performed by differential pulse voltammetry (DPV). The findings showed good sensitivity and selectivity [54]. For sensitive and rapid detection of *H. pylori,* an electrochemical nucleic acid-based biosensor was created by Revenga-Parra and coworkers by immobilization of a specific capture sequence of *H. pylori*. 3,4-DHS (N,N′-Bis(3,4-dihydroxybenzylidene)-1,2-diaminobenzene) was used as an electrochemical indicator for assessment of the hybridization event [55]. Another interesting study described measurement of the complementary target sequences of *H. pylori* over the range of 106–708 pmol with an acceptable detection limit. This method allowed the detection, without any need for a hybridization suppressor such as formamide in the solution, of not only a single mismatch but also its position in a specific sequence of *H. pylori*, owing to the selective interaction of a bifunctional ruthenium complex with dsDNA [56].

A novel disposable DNA biosensor based on thin-film gold electrodes was constructed for detection of *Salmonella*. In this study the hybridization event was detected using the ruthenium complex, [Ru(NH3)5L]2+, where L is [3-(2-phenanthren-9-yl-vinyl)-pyridine] as an electrochemical indicator. This work used an electrochemical platform based on 8 gold electrodes providing a multi-analyte detection system for *Salmonella, E. coli* and *Listeria* in a single sample. Therefore, it could have a wide range of applications in the analysis of different bacterial contaminants in food [57].

4.3. Immunosensors

Immunosensors rely on signal transduction based on the formation of stable antigen-antibody complexes. The future of immunosensors in clinical diagnostic laboratories can be crucial in terms of their ability to reduce costs. Although immunosensor technology remains largely at the stage of research and development. However, they may progress to take the place of established, clinical immunoassay systems such as ELISA.

An immunosensor is an analytic device that detects changes in the surface properties of the physical transducer in response to antibody-antigen binding. To date, several studies have been conducted on the use of immunosensors in the identification of fastidious bacteria. A number of these studies are summarized in Table 5.

Francisella tularensis (Ft) is a dangerous bacterium with a very low infectious dose. An anti-*Francisella* antibody (FB11) that recognizes the lipopolysaccharide found in the outer membrane of the bacteria was used for its detection. Identification was performed in by two-different methods. In the first, self-assembled gold monolayers bearing a carboxyl terminated bipodal alkane

Table 5
Recent immunosensors for fastidious bacteria.

Bacteria	Antigen	Technique	Sensitivity	Linear Range	Immobilization Method	LOD	Year-Ref
Francisella tularensis	LVS antigen	Piezoelectric immunosensor	10^4 CFU/mL	Not Reported	Covalent	10^4–10^6 CFU/mL	2007-[80]
Francisella tularensi	anti-Francisella antibody (FB11)	Microfluidics Electrochemical LVS 2	4 nA mL/bacteria.cm	Not Reported	Cross-linking	4.5 ng/mL_31 bacteria/mL	2014-[72]
Yersinia pestis	fraction F1	Magnetic-beads	25–300 ng/ml	25–300 ng/ml	Several methods	2.5 ng/ml	2007-[76]
Yersinia pestis	F1 antigen	lateral-flow immunoassay	10^8 CFU/ml	10^4 to 10^8 CFU/ml	Capture antibody	10^4 CFU/ml	2006-[77]
Haemophilus influenza	Chloramphenicol (CAP)	aptamer-polymer based electrochemical biosensor	0.102 mA/nM	0.1–2500 nM	Covalent	0.02 nM	2014-[81]
Chlamydia trachomatis	MOMP	Multiplexed nanoplasmonic biosensor	–	10–10^7 CFU/mL	Covalent binding	10^1–10^7 CFU/mL	2017-[74]
Rickettsia rickettsia	OMP H6PGA4_ RICRI,	Cyclic voltammetry-peptide based immunosensor	10 ng mL-1		–	2.59 μA	2017-[75]
Campylobacter jejuni	FlaA	Nanoparticle-based immunosensor	1.0×10^5 CFU/mL	1.0×10^3 to 1.0×10^7 CFU/mL	Covalent binding	1.0×10^3 CFU/mL	2010-[82]
Brucella	*Brucella* outer membrane protein (OMP)	Immune magnetic beads and quantum dots	96.15%	Not Reported	Covalent binding	cutoff value of 150.4	2017-[83]
Brucella	Smooth-A antigen	Optical fiber immunosensor	0.162/ln	5.00×10^6 to 1.95×10^3 cfu/ml	Photo-immobilization	Lower: 3.05×102	2009-[84]

thiol were used to covalently cross-link the anti-FB11 antibody. In the second method, F (ab) fragments of the FB11 antibody were created and chemisorbed onto the gold electrode surface [72]. In 2006, Meyer et al. designed a biosensor for the detection of *F. tularensis*. It used a sandwich immunoassay format with an antibody against Ft lipopolysaccharide (LPS) for immune capture. Magnetic beads coated by biotinylated-streptavidin were used as a label and immobilized onto ABICAPs polyethylene filters. The linear detection range of this biosensor was 10^4–10^6 CFU *F. tularensis* cells per mL [73].

The recent epidemic of sexually transmitted diseases (STD) has encouraged improved detection methods. A novel nanoplasmonic biosensor was described for simultaneous detection of the two most common bacterial STD infections: *C. trachomatis* and *N. gonorrhoeae*. A plasmonic microarray was supported on gold nano-hole sensor arrays that used optical transmission (EOT) for highly sensitive detection in a label-free configuration. The levels of the two bacteria were successfully detected and measured in a single phase microfluidics-based assay, without the need for DNA extraction or any amplification methods (Fig. 4) [74].

To detect Rocky Mountain spotted fever, due to *Rickettsia*, an immunosensor has been proposed using ultra-specific epitopes identified by a peptide microarray. Cyclic voltammetry was used for detection of the signal produced by interaction between the peptide and the antibody in blood samples [75].

In 2006, Meyer et al., developed a novel type of magnetic-bead based biosensor for the detection of *Yersinia pestis* using an antibody recognizing the antigen fraction F1 of these bacteria. The magnetic sensor platform offered reliable detection of *Y. pestis* with a linear detection range of 25–300 ng/ml *Y. pestis* antigen F1 and a detection limit of 2.5 ng/mL in human blood serum or buffer. The biosensor was small, simple, portable, and could be used in health applications and biowarfare analysis (Fig. 5) [76].

Another study for the detection of *Y. pestis,* showed a detection limit of approximately 10^4 CFU/mL and good linearity between the ratio and log CFU/ml of *Y. pestis*. The intra-assay and inter-assay variability was lower than 15%. Cross-reactivity with related Gram-negative enteric bacteria was not found [77].

A novel amperometric magneto-immunoassay was developed for selective determination of *Legionella pneumophila* SG1, based on

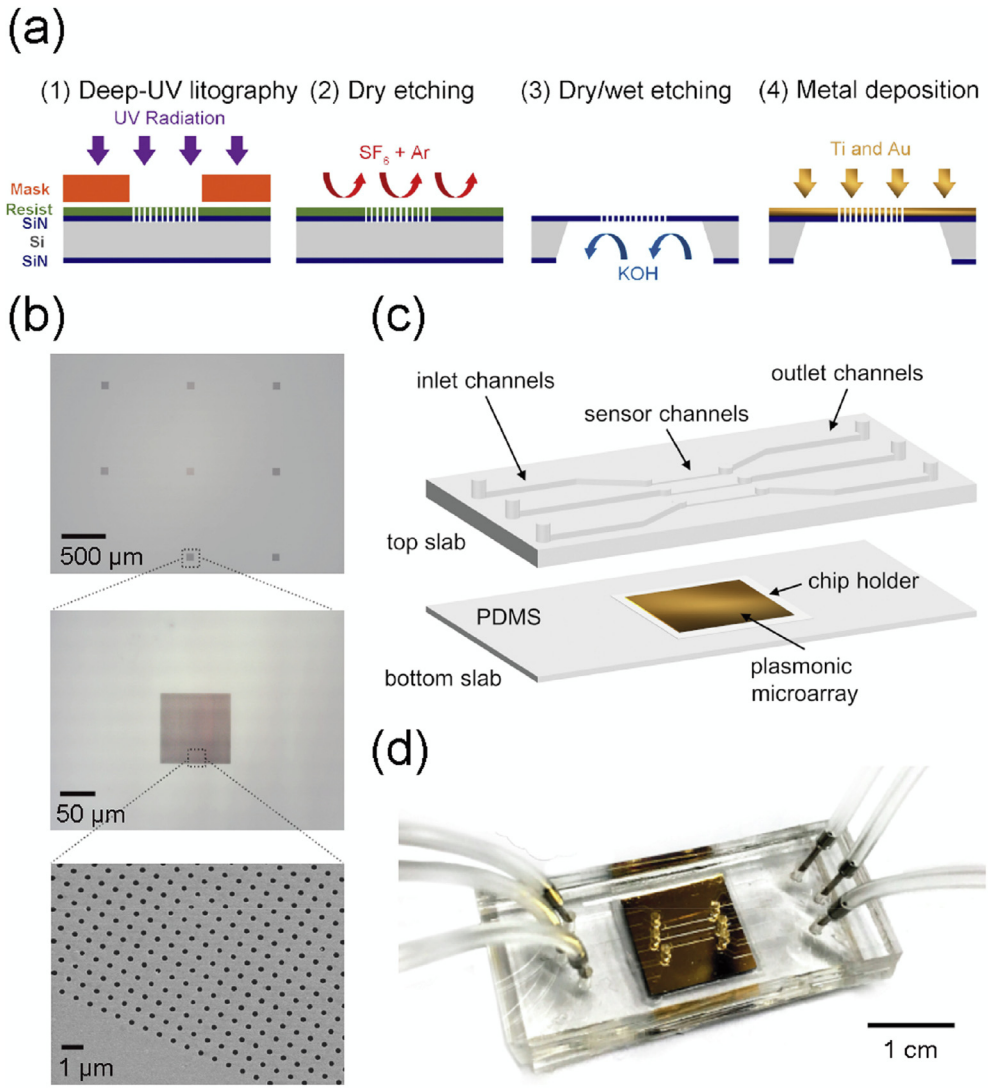

Fig. 4. Assembly of plasmonic nano-hole array and microfluidics integration: (a) Main fabrication steps of nanohole arrays on free-standing silicon nitride (SiN) membranes, (b) Optical microscope images of the plasmonic chip layout captured with a 4× objective (top) and 20× objective (middle); SEM image of the Nano hole array (bottom), (c) Graphic of the microfluidics system. The top PDMS slab was fitted in the 3 microfluidic channels with the inlet part, the sensor channels in interaction with the plasmonic chip and the outlet part. The bottom PDMS slab serves as chip holder and facilitates the binding to the top layer, (d) Depiction of the gold plasmonic chip combined with the PDMS microfluidics and the tubing for sample delivery [74].

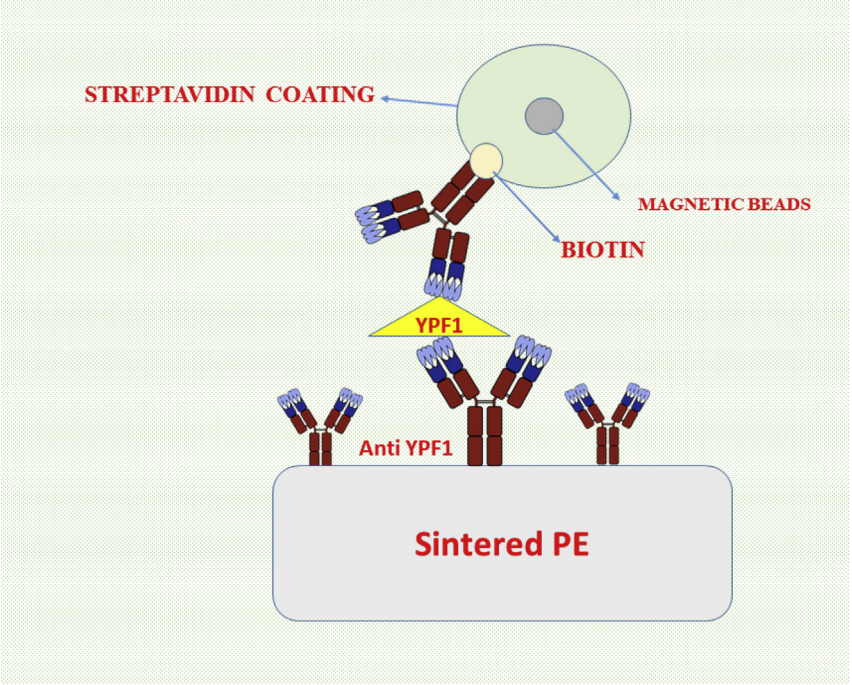

Fig. 5. Biosensor using antiYPF1 antibody to capture YPF1 antigen and detection with magnetic beads attached to biotinylated anti-YPF1 antibody. Binding to the magnetic beads resulted in an alteration of the induced magnetic field [76].

the use of core-shell magnetic nanoparticles and screen-printed carbon electrodes. Poly-dopamine modified magnetic nanoparticles (MNPs-pDA-Ab) were linked to a specific capture antibody (Ab) and incubated with bacteria. The antibody was labeled with horseradish peroxidase (Ab-HRP), and the subsequent MNPs-pDA-Ab *L. pneumophila*-Ab-HRP were affixed by a magnetic field onto the electrode surface. H_2O_2 production, in the presence of hydroquinone (HQ), was used as the transduction signal. The limit of detection, without any enrichment or purification, was 10^4 CFUs mL^{-1}. The biosensor exhibited good selectivity and the magnetic nanoparticles demonstrated good stability [78]. The enzyme (HRP) and electrode reactions involved in the amperometric detection of the mediated reduction of H_2O_2 with HQ at the SPCE are also shown Fig. 6 [78].

In 2018, an immunosensor was designed for brucellosis detection in various challenging environments and complex biological systems. In this study, poly-(ethylene glycol) (PEG) and hyaluronic acid (HA) were used to modify Fe_3O_4 @Au nanoparticles (NPs). This immunosensor was able to detect anti-brucellosis antibodies in 100% serum without suffering from any significant biological interference. This method showed high selectivity, sensitivity and good stability. A low limit of detection (LOD) of 0.36 fg mL^{-1} (3σ, $n = 13$) and a wide linear response range from 10–15 g mL^{-1} to 10–11 g mL^{-1} were obtained for the detection of antibodies in pure serum samples [79].

4.4. Micromechanical sensor

A new rapid way of sensing antibody–antigen interaction is micro gravimetric quartz crystal microbalance (QCM) analysis [85]. The QCM sensor is a general transducer used in immunological methods. It is commonly used for microbiological detection,

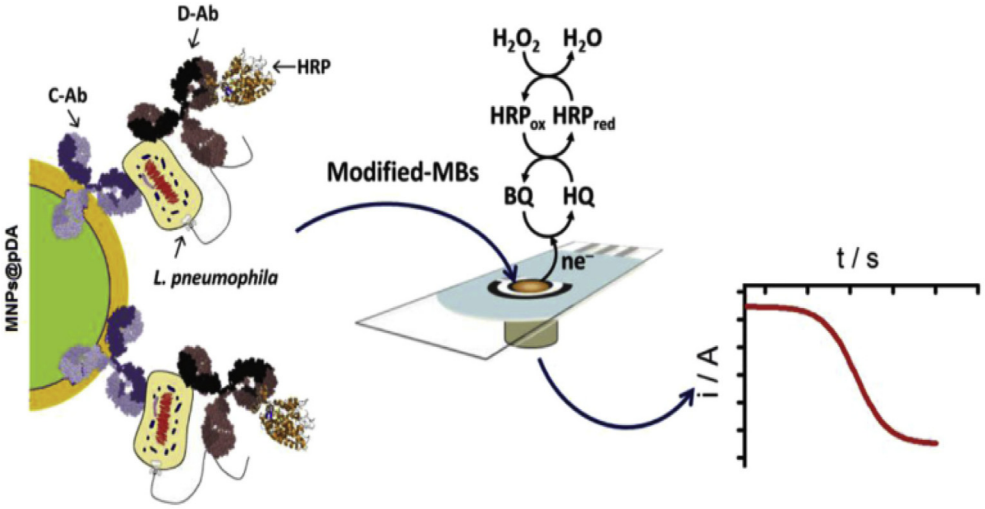

Fig. 6. Schematic illustration of the *L. pneumophila* sandwich magneto-immunoassay [78].

environmental monitoring, and food analysis [86]. The components for QCM biosensor operation are illustrated in Fig. 7.

Kleo and coworkers fabricated a novel biosensor based on QCM for detection of *F. tularensis*. They combined the QCM technique with a microfluidic system, which allowed the label-free online detection of the binding of whole bacteria to the sensor surface in a wide dynamic range of concentrations. Enhanced specificity and significant signal improvement, were obtained with antibody-functionalized gold nanoparticles and an enzymatic precipitation reaction [88]. In 2000, an original study developed a QCM based sensor for detection of *Salmonella* spp. The system was quite specific to *Salmonella* spp. and suitable for repetitive use after a regeneration step [89]. Similar to other biosensors, QCM sensors are simple, affordable and have high sensitivity. The paramount advantage of QCM is their ability to detect whole microorganisms without any extraction, purification or PCR.

Micro-cantilever sensors have attracted attention as a highly sensitive system for chemical and biological detection owing to their simplicity, sensitivity and their ability for label-free operation and real-time in situ monitoring. The sensing mechanism in the micro-cantilever is based on adsorption of the target molecules on immobilized receptors on the cantilever surface which changes the mechanical properties of the cantilever [90]. A micro cantilever-based pathogen detector system was developed for detection of *Salmonella enterica* strains. In this study, detection was achieved due to a change in the surface stress on the cantilever surface in situ upon binding of a small number of bacteria. This platform offers several advantages as well as high sensitivity and selectivity, low analyte volume, real-time detection and also the possibility for portable and implantable devices [91].

Many factors are important in choosing the ideal method for identifying fastidious bacteria. For example, rapid and simple methods are desirable in bacteriology. If the biosensor has the ability to detect a whole bacterial cell, it can be of great assistance in facilitating testing. Considering that it is possible to detect a complete bacterial cell using QCM and micro-cantilever sensors, they may be easier to use than other biosensors. In other words, in these types of sensors there is no need for bacterial lysis to liberate a specific DNA or antigen [92]. QCM and cantilever sensors are micromechanical sensors and, given their important advantages, they promise to attract a larger share of research in the near future.

4.5. Phage based biosensors

Bacteriophage-based probes have been combined with numerous analytical approaches to allow specific bacterial recognition. In 2010 a novel phage-based biosensor was described for detection of *Salmonella* spp. The biosensor was composed of a magnetoelastic (ME) resonator platform coated with filamentous E2 phage, engineered to bind with *S. typhimurium*. The results show that *Salmonella* contamination was detected on tomato surfaces painted with concentrations of at least 5×10^2 CFU/ml at a confidence limit of 80% or higher [99]. A novel phage-based biosensor was created for detection of *Campylobacter jejuni* by Singh, and coworkers. In this study the receptor binding protein (RBP) of Campylobacter bacteriophage NCTC 12673 was used for the specific capture of *C. jejuni* bacteria using RBP-derivatized capturing surfaces. The detection limit of the RBP-derivatized SPR surfaces was established to be 10^2 cfu/ml [100].

The advantages such as wide phage selection, host specificity, robustness and particularly a low reagent cost, promise to make phage-based biosensors ideal candidates to be considered as bacterial detection systems.

5. Comparison of traditional fastidious bacteria detection methods with biosensors

Among the traditional methods for identification of fastidious bacteria, microscopic observation has relatively low sensitivity and specificity. In addition, for highly pathogenic bacteria, staining may be unsafe for laboratory personnel. Also, with some bacteria, staining and microscopic observation requires special techniques, which will make the detection process lengthy and boring. Another major problem with the microscopic observation of bacteria is that a number of hard-growing bacteria are very small and can only be observed with specialized microscopes. For cultivation methods, despite moderate sensitivity and relative specificity, there is still the long time needed for detection of slow-growing species. Because of the quick and easy operation of serological methods, serology is one of the most widely used tests in microbiology; although the sensitivity and specificity of serology are often sufficient, the most important disadvantage of this test is the rate of false positive and false negative results. Molecular methods overcome many of the most important drawbacks mentioned above.

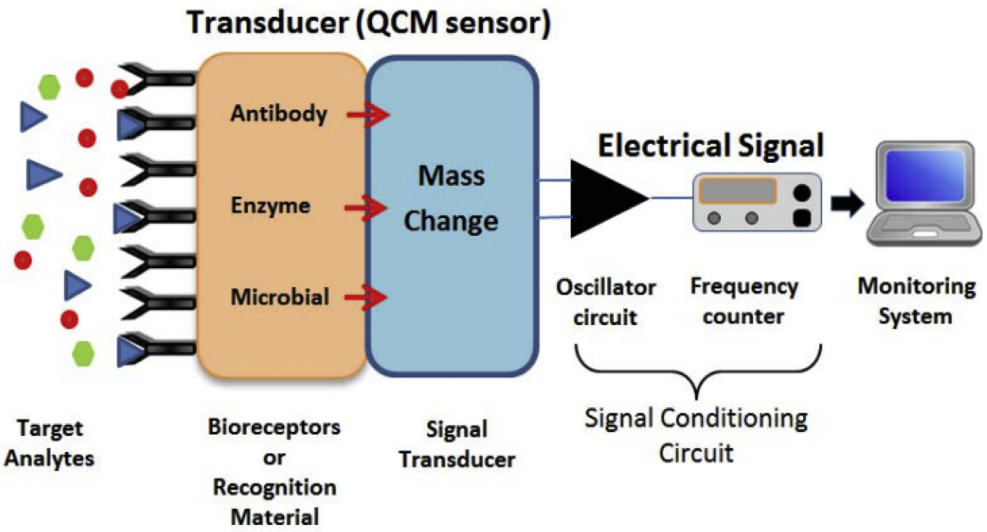

Fig. 7. Components for QCM biosensor operation [87].

Table 6
Micromechanical sensors.

Bacteria	QCM/Cantilever	Bioreceptor	LOD	Ref
Chlamydia trachomatis	QCM	Antibody	260 ng/ml to 7.8 μg/ml	[93]
Mycobacterium tuberculosis	QCM	DNA	5 pg of genomic DNA	[94]
Mycobacterium tuberculosis	Cantilever	Antibody	–	[95]
Mycobacterium tuberculosis	QCM	lipoarabinomannan (LAM)	10 μg/mL	[96]
Salmonella	Cantilever	Antibody	less than 25 bacteria	[97]
Francisella tularensis	QCM	Antibody	4.10^3 CFU/ml	[88]
Listeria monocytogenes	QCM	Antibody	10^2 to 10^6 CFU/ml	[98]
Salmonella typhimurium	QCM	Antibody	3.2×10^6 to 4.8×10^8 CFU/ml	[89]

Table 7
Comparison of sensitivity and specificity of routine diagnostic tests (serology and molecular methods) with biosensors for fastidious bacteria.

	Serology		DNA based		Biosensors		Ref
	Sensitivity	specificity	Sensitivity	specificity	Sensitivity	specificity	
Legionella	71%	–	High	93%	20pM	–	[5]/[101]/[62]
Brucella	7.5%	–	8.9%	–	79%	94%	[6]/[102]/[83]
Francisella tularensis	100%	99.6%	75%	–	4(nA.mL/bacteria cm LVS 2)	–	[7]/[103]/[72]
Leptospira	99%	86%	3 genome equivalents per reaction	–	1 genomic equivalent per reaction	–	[9]/[104]/[70]
Helicobacter Pylori	89%	98%	84%	71%	0.15 nM	–	[12]/[105]/[68]
Ureaplasma	20%	95%	37 CFU/mL	–	100 μgL	–	[13]/[106]/[107]
Treponema pallidum	–	95%	80.39%	98.40%	0 /5pM	–	-/[108]/[66]
Chlamydia pneumoniae	98%	–	76.5%	99%	107 CFU/mL	–	[74]/[109]/[74]
Yersinia enterocolitica	–	–	10 CFU	100%	$1.76 \times$ 10-12 mol L^{-1}	–	[76]/[110]/[45]

However, molecular methods, in most cases, are not cost-effective. In recent times serological methods, molecular based methods and biosensors are becoming more common than classical observation-based methods. The most important issue relative to detection methods is the sensitivity and specificity of the tests. These two very important properties are specifically compared in Tables 6 and 7 [16].

6. Conclusion

There are many available methods for identifying fastidious bacteria. The oldest methods include microscopy observation and culture. However these methods have several limitations mentioned above. Molecular detection methods are probably the best option amongst the traditional techniques. Molecular detection has high sensitivity and specificity and for slow-growing bacteria is much faster than cultivation methods, decreasing the time to detect fastidious bacteria from a few days to several hours. Despite the unique features and advantages of molecular-based methods, innovative modern alternatives often require skilled professionals, and molecular techniques require special equipment. Therefore, molecular tests are considered to be rather expensive methods. The emergence of biosensors provides powerful tools for detecting fastidious bacteria. DNA-based sensors with electrochemical transduction may be able to detect single bacteria and gene detection, using 16srRNA, is very important. As mentioned in the text, some biological sensors have been based upon identification of this gene. Despite the structural diversity of biosensors, these devices are relatively simple and working with biosensors can be simpler than molecular methods, while they still have high sensitivity and specificity. Additionally, the time it takes to identify difficult to grow bacteria is even shorter than with molecular methods. The basis for the use of genosensors and immunosensors is the identification of specific genes and antigens, so that the bacteria should be lysed to access them. Additionally, in conjunction with genosensors, it is also necessary to perform PCR to amplify the specific gene. To overcome these limitations, it seems that micromechanical sensors such as QCM and cantilever sensors could be a good choice because they have the ability to detect whole bacterial cells.

Acknowledgments

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