

# Chapter 20

## Nucleic-Acid Testing, New Platforms and Nanotechnology for Point-of-Decision Diagnosis of Animal Pathogens

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### Abstract

Accurate disease diagnosis in animals is crucial for animal well-being but also for preventing zoonosis transmission to humans. In particular, livestock diseases may constitute severe threats to humans due to the particularly high physical contact and exposure and, also, be the cause of important economic losses, even in non-endemic countries, where they often arise in the form of rapid and devastating epidemics.

Rapid diagnostic tests have been used for a long time in field situations, particularly during outbreaks. However, they mostly rely on serological approaches, which may confirm the exposure to a particular pathogen but may be inappropriate for point-of-decision (point-of-care) settings when emergency responses supported on early and accurate diagnosis are required. Moreover, they often exhibit modest sensitivity and hence significantly depend on later result confirmation in central or reference laboratories. The impressive advances observed in recent years in materials sciences and in nanotechnology, as well as in nucleic-acid synthesis and engineering, have led to an outburst of new in-the-bench and prototype tests for nucleic-acid testing towards point-of-care diagnosis of genetic and infectious diseases. Manufacturing, commercial, regulatory, and technical nature issues for field applicability more likely have hindered their wider entrance into veterinary medicine and practice than have fundamental science gaps. This chapter begins by outlining the current situation, requirements, difficulties, and perspectives of point-of-care tests for diagnosing diseases of veterinary interest. Nucleic-acid testing, particularly for the point of care, is addressed subsequently. A range of valuable signal transduction mechanisms commonly employed in proof-of-concept schemes and techniques born on the analytical chemistry laboratories are also described. As the essential core of this chapter, sections dedicated to the principles and applications of microfluidics, lab-on-a-chip, and nanotechnology for the development of point-of-care tests are presented. Microdevices already applied or under development for application in field diagnosis of animal diseases are reviewed.

**Key words** Lab-on-a-chip, Microfluidics, Nanotechnology, Nucleic-acid testing, Point of care, Veterinary diagnosis

### Abbreviations

AIDS	Acquired immunodeficiency syndrome
BTRP	Biological Threat Reduction Program
CNT	Carbon nanotube
DNA	Deoxyribonucleic acid

EIDSS	Electronic Infectious Disease Surveillance System
FIA	Flow-injection analysis
FMD	Foot-and-mouth disease
FMDV	Foot-and-mouth diseases virus
FRET	Fluorescence resonance energy transfer
GNP	Gold nanoparticle
HIV	Human immunodeficiency virus
HPAI	Highly pathogenic avian influenza
LAMP	Loop-mediated isothermal amplification
LATE-PCR	Liner-after-the-exponential PCR
MB	Molecular beacon
MNP	Magnetic NP
MWCNT	Multi-walled CNT
NASBA	Nucleic-acid sequence-based amplification
NP	Nanoparticle
PCR	Polymerase-chain reaction
PDMS	Polydimethylsiloxane
PMMA	Poly(methyl methacrylate)
PNA	Peptide nucleic acid
POC	Point of care
QCM	Quartz-crystal microbalance
QD	Quantum dot
RNA	Ribonucleic acid
rPCR	Real-time PCR
RT-PCR	Reverse-transcription PCR
SARS	Severe acute respiratory syndrome
S/N	Signal-to-noise ratio
S/V	Surface-to-volume ratio
SELEX	Systematic evolution of ligands by exponential enrichment
SPR	Surface plasmon resonance
SWCNT	Single-walled CNT
TADR	Threat Agent Detection and Response

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## 1 The Context of Disease Diagnosis in Veterinary Science, Medicine, and Practice

### 1.1 Current Situation

As in human medicine, disease diagnosis in veterinary medicine and practice is important for several reasons, including the issues of animal health and welfare, public health (especially in the case of zoonotic agents affecting pets or animals for human consumption), and economy (mainly related to the rules and barriers for the international trade of animals and animal products). In the veterinary field, it is important to consider fauna species other than cattle and farming animals (mainly large terrestrial livestock animals and fishes for aquaculture as well), like pets, captive (e.g., zoo, circus, and aquariums), and wild animals. All of these constitute underestimated sources of many human infectious diseases, especially of

viral origin. Animal or human contamination with a veterinary-relevant pathogen may occur through infected animals (live or dead) and infected animal products (e.g., bush meat, unpasteurized milk) or, to a lesser extent, through direct contact with the pathogen itself. In developing countries and regions, the impact of livestock diseases and outbreaks goes far beyond animal welfare and food safety. Too often, farm animals are also the only way of human and cargo transportation and also of income, as feedstock, as a source of manure and draft power, and as raw material for other industries (e.g., leather, shoes, and clothing) [1].

The advent of human immunodeficiency virus (HIV)/acquired immunodeficiency syndrome (AIDS) and other human immune depressing conditions has been a relatively neglected factor of enhanced susceptibility of humans to animal diseases, especially with respect to pets. Immune-depressed individuals or other vulnerable groups, such as children or the elderly, may even become susceptible to subtypes of zoonotic microorganisms that are usually harmless for healthy humans [2]. Interaction between domestic and wild animals may also provide an important via for indirect human contamination [3]. A particularly important group of zoonotic agents is that of enteric pathogens that are transmitted to and between humans and animals through the oral-fecal route, with subsequent dissemination to wastewater effluents. Outbreaks may occur upon contamination of surface waters and groundwater used for recreational and irrigation purposes and insufficient microbial removal of drinking water and/or of treated wastewaters, since many of these enteric pathogens are resilient to classical treatment and disinfection procedures [4]. They often contaminate water supplies in very small concentrations, hence hindering final disinfection and complicating detection as well [5]. Screening of enteric zoonotic agents has been proposed for pets cohabiting with immune-susceptible humans [6]. Very often, the presence of enteric viruses is detected indirectly, through bacterial indicators of fecal contamination, namely, coliforms and enterococci [5]. On the other hand, emerging “wild-type” zoonosis have been transmitted from animals to humans (e.g., SARS and West Nile virus) or after specific mutations that facilitate the “species jump” (e.g., HIV and H5N1 or H1N1 influenza) [7]. Concerning bioterrorism issues and depending on the nature of the infectious pathogen, the detection of potential zoonotic agents in animals should alert for an intentional release act [8]. A successful anti-bioterrorism strategy must not only account for the detection of the known pathogen but also of genetically modified forms. However, only a few potential veterinary pathogens fulfill the criteria for being considered effective bioterrorist agents. The group is composed by the viral agents of rinderpest, classical swine fever, African swine fever, avian influenza, Newcastle disease, Rift Valley fever, and foot-and-mouth disease (FMD) [1]. Among them, FMD and avian

influenza are especially important for their high prevalence in the world, zoonotic potential, and ramping spread. FMD is generally considered the most contagious viral disease in animals. Occasional occurrences in non-endemic regions are usually difficult and expensive to control [9]. Avian influenza is a viral disease with pandemic potential; pigs can act as intermediate hosts of influenza viruses between birds and humans. Bidirectional transmission of these viruses between humans and pigs is documented [7].

The diagnosis of air-borne diseases is challenging, with implications in disease control, especially in the case of outbreaks, and in individual case management. For these diseases, timely adoption of quarantine measures and massive antimicrobial therapy administration may be sufficient to contain an emerging outbreak, as long as the first cases are spotted in a short time. However, this may be impracticable in developing countries and regions, where effective routine surveillance flaws. Thus, a successful containment strategy must include rapid diagnosis through inexpensive and easy-to-use portable devices for in-the-field use [10]. As an example, it has been proposed that, within 6 h of the reporting of a suspicious FMD case, definitive detection should be made upon sample transportation to a national or regional laboratory, or else on the farm itself, and that, within 24 h of reporting, definitive identification of the infecting strain/subtype should be accomplished, immediately followed by vaccine production (from stored antigens) and distribution [1].

## **1.2 The Need for Point-of-Care Testing**

Following the trend of human medicine, the diagnosis of animal diseases has also undergone progressive decentralization, from central (reference) laboratories to in-the-field bioanalytical instrumentation, very often in resource-depleted regions and settings, even outside laboratory infrastructures. Essentially, this has been done through adaptation of conventional analytical methods and instrumentation to portable and automated devices, able to be handled by laboratory unskilled personnel (viz., veterinarian practitioners and farmers). Several nomenclatures have been given to these tests. They include the names “field tests”, “rapid tests”, “biosensors”, and “point-of-care (POC) tests”; this last term will be predominantly used throughout this chapter. Improved diagnostic tests are necessary for asymptomatic diseases, for diseases with misleading symptoms, for diseases requiring different treatments, or for diseases that, by their complex or costly treatment, require previous case confirmation. By contrast, these tests will likely have little or no impact for diseases which are easily recognized by their clinical symptoms and for which a syndromic treatment/approach is recommended. There is thus no clinical need to identify the causal agent. However, surveillance and control measures must be maintained for these diseases in order to deal with the risk of major epidemics [11]. It is among livestock animals that

disease epidemic outbreaks are more likely to occur, and with higher magnitude, given the physical proximity of many animals under intensive husbandry, the probability of pathogens to contaminate neighboring farms, and the relatively poor biosecurity conditions of most farms, but also in the case of subclinical diseases. As time is crucial when managing the early stages of infectious disease, an immediate preliminary positive result obtained by a POC test strongly argues in favor of precautionary and preventive measures in affected farms and surroundings in order to avoid further transmission, until a definite and more accurate result can be obtained in a reference laboratory [12]. Ideally, POC tests should go towards increased sensitivity, not only for early case identification but also for disease surveillance and epidemiological purposes, as infected animals may suffer and constitute infected reservoirs for disease spreading [11]. During the 2001 FMD outbreak in the United Kingdom, most of the livestock in infected and neighboring farms was targeted for culling during the first 24–48 h, based solely on clinical signs, which may be considerably doubtful, especially in sheep, the main species affected [13]. The time span between animal infection and the onset of clinical symptoms, and hence infection awareness, can take months, usually through rapid and silent spreading within and between herds, which might need to be destroyed [14]. However, indoor controlled studies with experimental infection of cattle with FMD virus (FMDV) showed a smaller-than-expected transmission fraction during the overlapping period between the incubation and the infectious period, suggesting that the importance of common preventive measures taken at endangered farms (viz., preemptive culling of cattle, often with severe economic downturn) has been overestimated [15]. For most viral diseases, since the onset of detectable viremia usually occurs at least 1 day before the onset of infection and/or presentation of clinical signs, efforts should be devoted to the development of new diagnosis methodologies and tools at preclinical stages. Historically, public funds have been used to compensate farmers for massive slaughtering of suspected diseased animals, but it has been argued that this may have generated permissive behaviors and disinterest from livestock industries and owners about transboundary control of cattle diseases [1]. In theory, POC tests are advantageous over laboratory-based tests if they can be used to detect infected animals before they become infectious, especially if this time window is short, as in the case of FMD. This advantage would apply if the time between collection of samples and receipt of results in the laboratory is, at least, 24 h. In the medium term, POC tests for cattle diseases will likely play an increasing role in the screening and triage of biological samples collected on the farm, as a primary support for decision-making during disease outbreaks [16], under a challenging context of permissive biological containment.

In veterinary medicine, POC tests have been largely used, for some decades, for the screening of physiological metabolites in blood and urine. Pathogen detection is a newer application, mainly driven by the increasing number of emerging outbreaks, including those with public health importance. Among these is, for instance, the potentially pandemic H5N1 avian influenza, as well as influenza viruses of putative porcine origin, as the human pandemic strain of 2009 [12]. POC tests have also been developed for important diseases of aquatic animals, using similar technologies to those used for terrestrial animals [17]. The most common layouts of commercially available devices rely on antigen detection in biological samples through affinity binding reactions with immobilized specific antibody probes. Nevertheless, despite the requirement for essentially the same performance features, regulatory approval and introduction of these devices into the market are understandably easier in the veterinary than in the clinical practice [18].

### **1.3 Difficulties Faced by Point-of-Care Testing**

Disease sub-notification remains a major problem for veterinarian health authorities; despite notifiable diseases are of obligatory reporting, too often farmers (and even veterinarians and diagnostic laboratories) ignore or delay the notification process owing to unawareness, minimization, or perceived lack of consequences, which may be aggravated when economic losses are anticipated. It is expected this scenario to worsen as POC tests become more readily available for utilization by farmers, especially in cases of delayed or misleading diagnosis based upon the clinical signs, as a result of disease underestimation or confusion with the signs of non-notifiable endemic diseases [12]. Of course, the interpretation of clinical signs itself and subsequent decision-making processes are compromised by the fact of farmers being unskilled personnel in health and veterinary medicine issues. Reducing the risk of non-reporting depends on tight cooperation and trust between veterinary authorities and livestock farmers and dealers [19]. In the livestock business, especially for important livestock diseases (e.g., classical swine fever), the risk of announcing a false-positive result is particularly worrisome, as it carries detrimental consequences in trade and export. Sanitary requisites on imported livestock for human feeding are very demanding and restrictive in most countries, leading to severe economic losses in cases of insufficient or inaccurate pathogen testing. Therefore, individual animal testing may be one of the main drivers for commercial development of POC tests, because a preliminary positive result with a POC test may prevent further and more expensive testing, while holding the herd being ineligible for export as well [12]. Some animal diseases are particularly contagious and able to spread rapidly. Many of them are considered transboundary diseases, meaning that their detection turns into the prohibition of livestock export and thus in economic losses. The combination of permanent surveillance

programs with improved techniques for rapid detection of emerging pathogen subtypes for efficient control of transboundary diseases has been claimed [20]. Unambiguous identification of circulating pathogen subtypes (*viz.*, genotypes and/or serotypes) is frequently a relevant epidemiological feature for the success of disease control programs. For low-prevalence diseases, the proportion of false-positive results is usually higher than for more prevalent ones. In addition, many of the current POC tests are less sensitive and specific than laboratory-based methods. Altogether, this means that, in order to achieve a desired level of performance and confidence in the results, sampling a large number of animals is usually required for POC-based testing of low-prevalent diseases [12]. In pets, the prevalence of many pathogens, particularly of zoonotic parasites, has been probably underestimated as a consequence, among other reasons, of using inadequate and inaccurate diagnostic tests and procedures, including limitations in sample collection and processing, which may directly result in false-negative results [21, 22] and, as such, putting the animals themselves and their owners at greater risk than currently assumed [3].

Intellectual property constraints frequently hamper the passage of these new devices from the proof of principle to the prototype and even commercialization levels. Another bottleneck is the high cost for final development of such devices, the reason why partnerships between research laboratories and biotechnological companies are usually established with such purpose. In this case, of course, the economic leitmotiv is much higher for high-impact diseases, especially those for which control of outbreaks is difficult and expensive. However, even for such diseases (*e.g.*, FMD), the only sporadic and unpredictable character of outbreaks might disincentive investments in individual testing; high demand usually only occurs by the occasion of an outbreak or suspected outbreak. Only a few tests for economically important livestock diseases have been developed and commercialized in developed countries, due to the limited market resulting from only infrequent diseases [12]. It seems that the most promising commercial segment for POC devices for veterinary pathogens concerns multiplexed platforms for a wide range of endemic and non-endemic pathogens [16], taking advantage of the relative simplicity and low cost of incorporating several additional targets in a multiplexed test. Indeed, veterinary health services eagerly lack rapid and simple diagnostic devices designed for in-the-field simultaneous detection, in a single sample, of a broader range of common pathogens [8], thus enabling the rapid gathering of field information on individual animals or whole herds disease status. Currently, simultaneous identification and eventual subtyping of many pathogens in a single test run is a province of central or reference laboratories and only available if the demanding entity (*e.g.*, person, laboratory, or agency) is able to afford to pay for such expensive testing. POC tests have

been also used for field differentiation between vaccine and field strains, especially in endemic regions where vaccination is permitted [12]. Suitable application of such differential testing at slaughtering settings to confirm prior livestock vaccination has been argued [1].

#### **1.4 Challenges for Point-of-Care Testing**

The ultimate goal of the use of POC portable devices by unskilled and unsupervised practitioners will likely pose enormous challenges for the overall bioanalytical process, from sample acquisition to post-analytical data storage, interpretation, and management. Bearing in mind that most of these devices are to be commonly used in remote and low-resource regions, uncontrolled and irregular reporting of results, in addition to test biosafety, especially when dealing with zoonotic agents, are major issues [23]. For these reasons, a balanced merging between in-the-field testing with remote data analysis and interpretation by competent clinical staff may be desirable. In this regard, telemedicine is an attractive system for healthcare improvement in developing countries, for allowing unskilled persons to provide useful healthcare in remote settings. This can be carried out by using miniaturized analytical devices with integrated hardware for image acquisition and software for storage of results. These results can be downloaded to hard copies or directly transferred online for a remote healthcare central unit for processing and interpretation by expert personal. Processed results can afterwards be returned to the tester, almost in real time [24]. Remote data from POC systems can still be incorporated in national disease surveillance and diagnostic systems, as the Threat Agent Detection and Response (TADR), developed under the Biological Threat Reduction Program (BTRP) of the US Defense Department's Defense Threat Reduction Agency. This system uses real-time polymerase-chain reaction (rPCR) for detection of pathogens. Another surveillance system, the Electronic Infectious Disease Surveillance System (EIDSS), contains a subsystem to report suspicious disease outbreaks in real time. The EIDSS is able to locate disease outbreaks through a geographic positioning device. This system aims to encompassing all available public and animal disease surveillance information. The EIDSS is able to report laboratory results from rPCR up to a few hours, depending on the distance. These systems will eventually enable a shift of paradigm in remote analysis, allowing world reference laboratories to simply handle and manage diagnostic information from pathogens and hosts based on biological samples remotely collected and analyzed, with subsequent reporting of results to the countries and regions of origin [1].

A full evaluation program of POC tests must include performance evaluation (validation), quality assessment (control and assurance), and standardization [25]. The performance of POC tests is highly dependent on the local conditions for their use,



on the epidemiology of the pathogen, and on the biology of the host [26]. Thus, the evaluation of such tests sets the particular ranges of in-the-field conditions in which they can be used [27], ideally under multicenter evaluation. Quality control assures correct functioning of the devices with prolonged use; usually, randomly selected units from a given production batch are compared to infer reproducibility. The quality assurance step demonstrates the readiness of the tests to be introduced into the routine diagnostic practice. Standardization is needed in POC diagnostics for veterinary applications, as well as new rules to compare results obtained from the two different methodologies—the POC and the laboratory-based method, respectively—which implicates in the final (accepted) diagnostic result [23, 28]. Proper validation and standardization of many POC tests frequently lack, in part because these often correspond to in-house rather than to well-standardized assays and techniques. It is thus important that national or reference healthcare entities establish quality assurance programs that guarantee the production of reference materials and protocols for effective quality control of rapid diagnostic tests [29]. For the clinics, a set of guidelines for standardization of POC blood-based tests has already been established [30]. Hopefully, in the future, this will also be useful to inspire standardization procedures in new diagnostics for veterinary medicine and practice.

POC tests hold great promise to shift the paradigm of veterinary diagnostics, particularly for field situations, and in cases in which the biological sample is easy to collect and process. However, its advantageous use presumes the availability of sufficient manpower to survey enough animals on high-risk premises, which remains a burdensome challenge. The decision of whether using them or not to manage animal infectious diseases depends on biological and epidemiological factors, on the specific circumstances in which clinical signs arise, on cost-benefit issues (taking into account the costs for deployment, including equipment and reagent storage), and on the relative performance compared to diagnostic tests in reference laboratories. The possibility of sending suspect samples to a central laboratory within 24–48 h has probably discouraged the use of POC tests in some past situations in Western countries, e.g., under medical suspicion of highly pathogenic avian influenza (HPAI); from another point of view, this strengthens the idea of a promising use of these tests in remote regions, far away from laboratory settings. Once POC tests have equivalent analytical sensitivity and specificity to laboratory-based tests, it may be foreseen, on the basis of a POC test alone, to take actions beyond simple restriction/quarantine on suspect premises [12]. Transboundary livestock diseases are probably among the major concerns in veterinary medicine. It has been proposed that an efficient governmental control strategy of these diseases should include legislation enforcing, namely, (a) disease screening in

people, conveyances, and goods arriving from other countries, in a reasonable extent, according to previously defined performance benchmarks, including possible use of robotic automated devices; (b) higher extent of screening for people, conveyances, and goods arriving from endemic countries, even reaching exhaustive screening when such countries are recognized as not making acceptable progresses in disease control; and (c) the need to develop tests to certificate animal product's region or country of origin, at the point of importation [1]. These points certainly constitute an immense and valuable fountainhead of opportunities for commercial attraction-driven development of new POC devices in the veterinary field.

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## 2 Nucleic-Acid Testing

### 2.1 *Advantages for Disease Diagnosis*

Early disease diagnosis not only prevents or minimizes potential animal suffering but also the risk for infection transmission among susceptible hosts [31]. In contrast, delayed diagnosis carries an increased risk for severe symptoms and complications, ultimately leading to longer suffering, increased toxicity due to accumulated pathogen load and/or virulence effects, and a higher risk for acquisition of drug resistance [12]. Antibody-based detection requires a sufficient level of antibodies to be produced for successful detection, which usually happens only some days after exposure to the pathogen. In this regard, specific detection of pathogen nucleic acids in biological samples by PCR-based methods may be more suitable, since the pathogen genomic material is present in the host from the very beginning of infection and even very little amounts are detectable before the onset of immune response markers or clinical or veterinary signs. Moreover, methods based in nucleic-acid detection are able to detect both live and dead pathogens, an advantage for disease diagnosis [1], especially when time is crucial for disease containment and control. In conventional methods (e.g., cell culturing and serology), pathogen detection strongly depends on the sample quality [13], leading more easily to false-negative or irreproducible results.

Deoxyribonucleic acid (DNA) is a particularly suitable material for biosensing owing to unique characteristics, including the ability for highly specific and mutual recognition between a short immobilized oligonucleotide (probe) and a longer-sized genome and high physicochemical stability. Unlike enzymes and antibodies, DNA forms biological recognition layers easily synthesizable and readily reusable after thermal heating [24]. DNA is also prone to very specific manipulation and processing precision by ligases, nucleases, and other enzymes [32] and is the most easily copyable biomolecule, through PCR and similar techniques

of amplification, which is also an enormous advantage for the development of portable and miniaturized diagnostic devices. In addition, DNA micro- and nanoarrays are more suitable than protein counterparts for direct synthesis onto a chip surface, without the need to produce and purify the ligands [33]. Most of these features of DNA are also extensive to ribonucleic acid (RNA). Indeed, nucleic-acid (molecular biology) testing comprises genetic analysis (DNA level) and functional genomics (including mRNA, non-coding RNA, and microRNA). In particular, functional genomics dedicates to the identification and analysis of specific RNA expression patterns (profiles) associated with particular experimental or clinical conditions. DNA (and RNA) microarrays and quantitative PCR have been the most common techniques used with this purpose [34]. DNA microarrays (DNA chips) are usually produced in the form of highly dense arrays printed on a silicon or glass chip, coated with different probes for simultaneous detection of multiple DNA-target sequences [35]. Current microarrays can detect in the order of  $10^4$  nucleic-acid sequences on a single chip [1]. The Virochip is particularly well succeeded in this regard; it is a panviral DNA microarray platform, able to detect known viruses and new viruses related to known viral families, in a single assay. Its high robustness has been confirmed with viruses of high genetic variability, as the swine virus. This platform is especially useful for detecting viruses for which there are no available reverse transcription-PCR (RT-PCR) assays [7]. Nevertheless, microarrays do not seem promising for the POC use, being too expensive, complex, and bulky [24]. Another peculiarity of DNA-based detection is the superior ability to differentiate strains from the same organism, especially when isolated from different geographical locations. This is useful not only for pathogen identification but also for epidemiological studies. As an example, the significant differences among the nucleic-acid sequences of the different serotypes of FMDV (as in other viruses) and the likelihood for the occurrence of typing errors during viral RNA replication demonstrate how the virus can rapidly evolve in nature [36], which constitutes an enormous challenge for successful viral identification. In influenza viruses, genetic reassortment is able to generate novel subtypes whose cell surface antigens might no longer be recognized by preexisting antibodies (antigenic drift) [37]. The potential for pandemics caused by novel highly pathogenic subtypes strongly stresses the need for specific subtype identification through nucleic-acid testing. Of course, the drifting in influenza viruses, responsible for the sudden onset of dominant subtypes during a yearly season, requires not only rapid development and validation of suitable molecular probes for nucleic-acid tests but also the unobstructed use of such tests. Unraveling the pathogen subtypes circulating in a given region may assist in designing

tailored vaccines and disease containment strategies, as well as tracking outbreaks' sources [38].

## **2.2 Nucleic-Acid Testing in the Point of Care**

Most of the current POC tests for human and animal medicine still rely on antigen/antibody bioaffinity reactions, but they are usually handicapped by relatively small sensitivity. Thus, there is a trend for shifting towards more sensitive nucleic-acid detection, usually based on DNA/DNA hybridization schemes. A major challenge for detection of pathogen nucleic acids in host body fluids, apart their scarcity compared to antigenic proteins, is the confinement inside pathogen cells, surrounded by hard biomembranes and cell walls. Consequently, the nucleic-acid levels present in host fluids may be too low for successful diagnosis without prior target amplification by PCR-based techniques. For blood infections in humans, the amount of human genomic DNA can be  $10^{14}$  times higher than that of pathogen DNA [39], an important challenge in terms of sensitivity and selectivity. For POC testing, this constitutes a challenge for the miniaturization of the blood sample preparation step, especially in the case of gram-positive bacteria, whose cell walls are thicker and more rigid than those of gram-negative bacteria. The majority of available POC systems for clinical and veterinary applications require off-device sample preparation (including microorganism concentration and nucleic-acid extraction). This is a traditionally cumbersome and time-consuming step, especially for in-the-field testing, but newer processes, including filter paper capture of nucleic acids and automated extraction procedures and kits, have been developed to simplify such task [40]. In parallel with POC device development, considerable efforts have been spent in the simplification and robustness of sample preparation, in order to minimize manual handling, thus reducing cross-contamination and the effects from potentially interfering substances present during the nucleic-acid amplification assay. Of note are the technical difficulties inherent to the analysis of matrices such as feces, semen, and decomposing tissue or the detection of *Mycobacterium tuberculosis* from saliva [12]. For these cases, it is necessary to incorporate, in POC devices, systems that are able to suppress such interfering substances and that reduce handling prior to the nucleic-acid amplification step.

PCR has been, by far, the most widely employed technique for nucleic-acid amplification in laboratory analysis but also in POC tests. Many similar techniques have been originated from the basic PCR principles, including nucleic-acid sequence-based amplification (NASBA), targeted for direct RNA amplification, thus precluding the need for a previous reverse-transcription step. The development of this panoply of techniques for pathogen detection has been possible due to the increasing availability of whole genomic sequences; a remarkable case in veterinary science is the current possibility of rapidly distinguishing severe acute respiratory

syndrome (SARS) from other circulating coronaviruses [41]. Nevertheless, PCR is prone to false-positive results, as a result of unwanted amplification of contaminant nucleic acids. Proper quality management of these issues requires using adequate controls for each stage of the testing process [12]. For RNA analysis through RT-PCR, storing biological samples at very low temperatures is crucial, owing to the high lability of RNA genomes. Innovative lyophilized reagents with enhanced sensitivities have significantly improved the operational conditions for POC disease diagnosis [42]. Moreover, the need for improved stability of RNA used as an internal positive control, especially for real-time RT-PCR assays, has led to the search for new RNA sources. Another approach for improving RNA assays is the use of minor groove-binding probes which, by being short sized, are suitable for multiplexing and for targeting sequences of highly variable genomes [20]. Nevertheless, PCR-based techniques remain challenging for POC pathogen diagnosis in many endemic regions of the world. Even in human medicine, however, RT-PCR, for instance, is only used for clinically important specimens, due to the complexity and time demanding of this technique [43]. Linear-after-the-exponential PCR (LATE-PCR) is another PCR-based technique for detection of RNA. Probably its most known application is in FMDV subtyping, to tackle the difficulty of targeting all field strains with novel sequence mutations, even those in target sequences for which degenerate primers are being used [44]. The amplification process can begin with as few as a single DNA molecule and abundantly generates amplified product over a broad temperature range. The technique employs primers that hybridize to conserved genomic regions and a mismatch-tolerant probe able to target different variable sequences, depending on the temperature. Thus, the technique is especially convenient for detection of RNA viruses [36].

Among the vast diversity of available nucleic-acid amplification schemes and devices, both at the proof-of-concept and commercial levels, the technique of loop-mediated isothermal amplification (LAMP), already widely used in laboratory analysis, seems quite promising for application in POC systems for pathogen detection and identification. In remote PCR-based nucleic-acid testing, power supply is a keystone for device operation, although the use of rechargeable batteries is expected to circumvent this problem, by avoiding the need for an external power source [42]. Advantageously, LAMP does not require thermal cycling for the amplification, rather making use of a simpler power source compared to a PCR thermocycler. Such operation at constant and low temperatures permits LAMP to be incorporated in polymer-based microdevices instead of more traditional and expensive materials, like glass and silicon that, for being temperature-resistant, are required for PCR-based devices. Unlike PCR, LAMP does not require a denatured template for amplification, and given the usual

abundance of generated DNA, the amplified product can often be visualized directly [13]. For viral detection, in particular, the technique has shown sensitivity at least equivalent to PCR [45] and even to rPCR [46]. Coupled to easier sample preparation procedures, these features make LAMP inherently simple and hence especially suitable for POC applications [47].

POC tests based on nucleic-acid amplification potentially allow early detection of latently infected animals on targeted high-risk premises, with some examples already being applied in field situations [48]. However, this would require sample collection and testing from many suspect herds, which will require too demanding manpower in the course of an outbreak. This is probably why existing tests have not been adopted in contingency plans in many countries, although the issue remains controversial [12]. During the FMD outbreak in 2007, up to 269 samples were analyzed each day [38], but larger outbreaks would require higher-throughput testing. Of course, from a certain point above, it is admissible that central laboratories would become overloaded and that true POC devices, able to be manipulated by farmers themselves, would be probably the best (or even unique) solution. This, however, by the reasons pointed above, is still far from reality, although the unstoppable technological advances in this field, coupled to new paradigms for decentralized detection, will more and more shorten the present distance to that goal.

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### 3 Methods for Signal Transduction in Biosensors and Point-of-Care Devices

For bioanalysis with prototype biosensors and POC devices, three main types of signal transduction mechanisms have been used: electrochemical, optical, and microgravimetric (mass sensitive). They are well known and have been extensively used in research laboratories for analytical chemistry, holding great promise for new prototype and fully developed bioanalytical and diagnostic micro-devices. Among microgravimetric methods, quartz-crystal micro-balance (QCM) is the most common; it usually relies on the use of a piezoelectric crystal whose fundamental resonance frequency changes upon successive immobilization of a biorecognition probe and its target. However, the difficulty of miniaturization, the high cost, and the complex influence of multiple interfacial parameters on the sensor response (especially in liquid phase) have hampered wider development of POC devices based on this technique. The applicability of electrochemical methods for nucleic-acid analysis has greatly benefited from the emergence of solid electrodes [24]. Electrochemical-based analytical devices can be relatively simple, rapid, less costly, low-power demanding, and amenable for miniaturization and mass production through standardized micro-fabrication techniques [49].

Optical detection has been the most widely employed transducing principle in biosensing, partially driven by the unending advances of optic applications in telecommunications and information systems. Furthermore, the very high frequency of optical signals is appropriated for the enormous amount of information that can be carried by optical systems and devices [24]. Conventional optical microscopy has been reliably used for detection and imaging of infectious pathogens (at both cellular and molecular levels), integrated with research and development of new POC diagnostic devices [50]. Within optical detection methods, chemiluminescence and fluorescence have been the most widely used for their versatility of designs and applications. Imaging methods usually require bulky and expensive microscopes and camera-equipped microscopy systems, i.e., in off-device formats. Such equipment is obviously unsuitable for POC diagnostics. The innovative technique of optofluidic seems thus promising in this regard, as this high-throughput and high-resolution on-device technique does not make use of such components [51]. In optofluidic devices, the sample flows through a metal film-etched array with submicron apertures for imaging, onto a plastic platform.

An intrinsic limitation of conventional optical detection is the interference between closely spaced light waves. Understandably, this is especially limiting for the design and performance of miniaturized devices for multi-analyte detection and identification. However, optical transmission through minuscule structures gained a new impulse with the technique of surface plasmon resonance (SPR), in which light waves are directed to the interface between a metal and a dielectric. The technique is label-free and the immobilized probe can be easily reused, although sensitivity must still be improved [24]. Moreover, it faces the challenges of other optical techniques for POC diagnosis, namely, the high cost, instrumental complexity, expensiveness of optical components, and difficulty for miniaturization/portability and mass production. The advent of integrated photodiode detectors is promising for the development of cheap, sensitive, easy-to-use, and easy-to-fabricate fluorescence-based diagnostic microdevices [52]. A remarkable milestone might be the shift from current fluorescent-based detection methods to naked-eye (visual) recognition, which would considerably reduce complexity and costs. These readouts can be recorded as digital images and then transmitted to remote clinicians through trivial and inexpensive telecommunication devices (e.g., digital cameras and scanners); some current examples include the detection of glucose, pH, and proteins [53]. It is expected that future POC diagnostic devices will become connected to wireless communication and information systems through camera-equipped mobile phone networks coupled to web databases, for diagnostic imaging and telemedicine, especially in remote and resource-limited regions and settings [54]. Remote monitoring will not



constitute a valuable or even indispensable way for analysis when in-the-field conditions do not allow doing that, but also an approach to improve quality management for tests conducted in the field.

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## 4 New Platforms for Point-of-Care Disease Diagnosis

### 4.1 *Microfluidics*

Multiplex PCR and DNA microarrays have been among the most commonly used techniques for the detection and identification of infectious pathogens. Multiplex PCR is frequently limited to the analysis of only a few target genes, owing to interferences between different primer sets in the same reaction vessel. DNA microarrays are usually too expensive, labor intensive, and complex for POC applications; moreover, the microarray assays are usually lengthy due to the slow diffusion-limited hybridization kinetics. On the other hand, lateral-flow devices employed for POC diagnosis usually have relatively low sensitivity; moreover, in the most common format (immunochromatographic format, corresponding to the well-known test strips), they do not provide genetic information, thus hindering the assessment of eventual pathogen subtypes and/or drug resistance markers. In general, the number of targets that can be simultaneously probed in lateral-flow assays is low, thus increasing the cost per assay and limiting the throughput. Also, their degree of multiplexing usually does not reach that of microarrays [55].

The last decade has witnessed tremendous advances in microfluidic sciences and applications for bioanalysis. Microfluidic devices basically consist in a set of microchambers interconnected through microchannels, imprinted onto a suitable solid platform. After injection into the system, the liquid biological sample flows throughout the hydrophilic inner walls of the chambers and channels for final analysis. The flow is usually controlled automatically by mechanical valves. A primary advantage of microfluidics for the development of miniaturized diagnostic devices, especially for POC usage, is the requirement for only very small sample volumes, which permits to save reagents (*viz.*, probes and labels), but also gaining time and sensitivity, as a consequence of the enhanced binding kinetics (unlike in bulky reaction media). It also provides lower power consumption and lower operating costs. Moreover, since the overall analytical process is automated, sample losses and contamination due to human handling can be minimized, in parallel with improved multiplexing capabilities. The confinement and automation of the process also reduces errors due to human manipulation and increases biosafety. In microfluidic devices, the occurrence of nucleic-acid hybridization in solution and the enhanced aqueous mixing allow surpassing the kinetic barriers that slow affinity reactions due to limited diffusion in traditional



microarrays [53]. Recent developments in microfluidic technology have pointed towards integrating traditionally off-chip processes into the microdevices. Indeed, significant reduction of bulky, complex, and expensive equipment is mandatory for POC and in-the-field diagnostic applications.

Traditional microfluidic devices usually rely on pressure-driven flow (through a syringe pump or pressure injector) or electroosmotic flow, but these techniques require external bulky and complex equipment, like a power supply. For POC applications, more suitable methods for fluid delivery have been developed, namely, mechanical pumping, capillarity, and light-driven motion [53]. Compared to conventional injection systems, as flow-injection analysis (FIA), microfluidics enables achieving lower detection limits, as a result of enhanced probe/target binding kinetics [56] and of time-dependent target pre-concentration in microchambers. Droplet-based schemes offer a significant improvement in microfluidics, due to the ability to independently control each free droplet containing the target under analysis. In this way, each single droplet functions as a pump, valve, mixer, solid-phase extractor, and thermocycler, thus greatly simplifying the assay design [10].

The first microfluidic devices were manufactured as glass or silicon platforms, but the high cost of these materials led to the widespread use of polydimethylsiloxane (PDMS) for such purpose. However, some properties of PDMS limit its use for biosensing in microfluidic devices, e.g., hydrophobicity and swelling/disintegration in organic solvents. In order to prevent some of the limitations of PDMS, other materials have been used, like paper and thermoplastics. These materials, as well as their fabrication process, are cheaper than PDMS, glass, and silicon, making large-scale fabrication more feasible. This low cost also encourages disposability, an important biosafety asset when dealing with infectious agents, and a way to avoid cross-contamination of biological samples. Moreover, these materials are lightweight and hence easy to transport in the form of portable devices. Finally, their fabrication does not require clean-room settings. Thermoplastics are attractive materials for simple, low-cost, and reproducible fabrication of POC devices by well-known replication molding techniques (e.g., injection molding or hot embossing). An example is poly(methyl methacrylate) (PMMA), which is mechanically stable, is optically transparent, and has an easily modifiable surface. Another example is polycarbonate, whose lower thermic conductivity in comparison with glass or silicon turns it amenable for high-temperature PCR applications [53]. Patterned paper-based microfluidic devices are valuable alternatives to test strips. Advantages of paper include its abundance, easiness to use (e.g., clean rooms or other complex and expensive infrastructures for its manipulation are unnecessary), low cost, disposability, and easily patterned with polymers and other molecules by conventional printing techniques

(e.g., photolithography, wax and inkjet printing). Its common white color is particularly suitable for the use of colored substrates in quantitative colorimetric tests. Moreover, unlike in test strips and conventional microfluidics, its porous nature allows capillarity-driven fluid wicking and flow through its structure, without the need for external pumps and pressure source. New three-dimensional structures will enable a vertical flow to be added to the sole lateral flow of two-dimensional devices, in higher complex structures than those presented by plastic frames. In this way, they may exhibit improved abilities for initial purification and concentration as the liquid wicks throughout the inner layers of the device, until final detection [57].

Many current microfluidic devices still rely on complex and bulky laboratorial (off-chip) equipment for fluid delivery and control, making their use impractical in field situations, e.g., by emergency response teams [53]. Other drawbacks faced by microfluidic systems include biomolecule adsorption to microchannel walls, inaccurate control of temperature, liquid evaporation due to heat-generating components, and bubble formation [5]. In addition, future developments in microfluidic platforms may eventually benefit from materials with improved analytical performance [58], as well as simplicity and cost-effectiveness.

#### **4.2 Lab-on-a-Chip**

The processes of sample purification and pre-concentration are usually the most complex and cumbersome in biological analysis. In molecular diagnostics, this is particularly true for nucleic-acid isolation procedures, which still remain too complex for in-the-field use. One of commonest requirements is a centrifuge, which obviously hinders the integration of such detection schemes within miniaturized analytical devices. In many diagnostic microdevices, such procedures are carried out off-chip, prior to the detection step itself. Moreover, they strongly depend on the characteristics of the specific biological sample and require relatively high volumes of raw sample for downstream nucleic-acid amplification. In the last years, lab-on-a-chip devices have emerged as second-generation chips, essentially based on the concept of microfluidics. By suitable integration of modules for sample processing and analysis in a single device, they offer enhanced flexibility and discriminatory ability over conventional diagnostic methods and devices. Yet, minimizing and miniaturizing the whole sample preparation procedure towards easiness to use and true POC remains challenging and a relatively underestimated task in the development of POC tests [5]. Compared to conventional detection techniques, lab-on-a-chip devices allow the integration and automation of all (or nearly all) steps for sample processing and analysis; the confinement of the bioelement under measurement in a predefined region of interest facilitates its detection and eventual quantification from very small sample volumes. This, in turn, reduces background noise and

hence increases sensitivity [53]. In lab-on-a-chip devices, minimization of the chemical interferences that result from tight spatial confinement is usually envisaged, enabling miniaturization and highly sensitive detection. The high mass transfer rate thus achieved is due to the low diffusional distance and high surface-to-volume ratio (S/V) [59]. Traditional cell analysis and processing, including cell sorting, cell/serum separation (for immunoassays), and cell lysis (for immunoassays or nucleic-acid amplification), can be adapted to POC schemes [53]. Some principles commonly used to separate and concentrate cells within POC devices are cell size, labeling (fluorescent or magnetic), electrophoretic mobility, and cell adhesion [60]. For analysis of real, biological samples, the detection of highly virulent pathogens presumes the ability of detecting very few cells from large sample volumes and, concurrently, washing out sample contaminants (e.g., other cells), in order to prevent, for instance, downstream PCR inhibition and microchannel clogging [61]. In particular, environmental water samples may contain certain substances, like humic acids, that inhibit PCR reactions, able to drastically decrease the resulting signals. The presence of contaminants in biological samples may be particularly troublesome in microfluidic assays for veterinary applications.

A particular advantage of bioanalysis with lab-on-a-chip devices concerns the processing of pathogen RNA. Owing to the confined environment of the purification steps and to the requirement for minimal user handling, the probability for RNA contamination and degradation by ubiquitous RNases is highly decreased [62]. Schemes employing nucleic-acid extraction through detergents rather than high temperature-operating lytic enzymes coupled with isothermal (e.g., LAMP) instead of PCR and RT-PCR amplification processes are also preferred in this regard. In this way, the concept of microfluidics might be able to address a recurrent gap in veterinary literature—the lack of information about RNA quality and integrity, which further complicates the standardization of RT-PCR procedures [34]. Several commercial multiplexed POC tests for diagnosis of influenza and other respiratory viruses are already available, as the FilmArray RP (BioFire Diagnostics, Salt Lake City/UT, United States), the xTAG RVP (Luminex, Toronto, Canada), and the Xpert Flu A Panel (Cepheid, Sunnyvale/CA, United States), consisting in closed systems that include sample preparation and detection by integrated RT-PCR [63, 64]. However, these systems may not be able to detect low viral load specimens, as is the case of the Xpert Flu A Panel with some important avian influenza viruses. In addition, economic constraints still restrict full applicability of some of these tests in POC testing. For the Xpert Flu A panel, it was estimated a cost of 45 euros per test, compared to 15 euros for the antigenic tests [63]. Current lab-on-a-chip devices, despite being simpler than conventional laboratory

**Table 1**

**Illustrative works described in the literature employing nucleic-acid amplification methods for the detection of animal pathogens. POC tests are those claiming, at least, one of the following characteristics: lab-on-a-chip; microfluidics; portability**

Target pathogen(s)	POC		Amplification method	LOD or sensitivity	References
	Yes	No			
African swine fever virus		×	LAMP	≤330 copies	[67]
Avian influenza virus (H5N1)	×		RT-PCR	–	[10]
Avian influenza virus (H5N1)	×		rRT-PCR	98 %	[42]
Influenza virus	×		LAMP	90.9 %	[43]
Influenza A viruses	×		RT-PCR	400–5,000 viral particles/ml	[63]
Influenza A virus (H1N1)	×		RT-PCR	–	[62]
FMDV	×		rPCR	10 <sup>-9</sup> dilution	[9]
FMDV		×	RT-LAMP	10 copies	[13]
FMDV	<sup>a</sup>		RT-LATE-PCR	10 copies (100 %)	[38]
Respiratory viruses	×		RT-PCR	82.2–100 %	[64]
SARS-CoV		×	RT-LAMP	0.01 PFU (100 %)	[41]
Swine viruses (H1N1 and H2N3); influenza A (flu A; seasonal H1N1; pandemic H1N1)	×		LAMP	<10 copies/μl	[65]
<i>E. coli</i> (O157 and K 12)	×		PCR	0.2 CFU/μl	[61]
<i>S. aureus</i> (MRSA) and FMDV	×		RT-LAMP	17 copies	[40]
<i>E. coli</i> ; <i>B. subtilis</i> ; <i>E. faecalis</i>	×		RT-PCR	10 <sup>2</sup> to 10 <sup>4</sup> CFU/ml	[66]
<i>B. anthracis</i> ; <i>Brucella</i> spp.; <i>F. tularensis</i> ; <i>Y. pestis</i>	×		rPCR	10–100 fg	[8]
Aquaculture pathogens ( <i>S. agalactiae</i> ; koi herpes virus; Iridovirus; <i>A. hydrophila</i> )	×		LAMP	20 copies	[68]

<sup>a</sup>Pilot test adaptable to POC (under development)

analytical apparatus, still require complex and somewhat expensive fabrication procedures onto plastic or glass substrates, thus severely limiting their affordability and availability by poor countries. Table 1 displays some literature references about proof-of-principle schemes and POC devices for detection and identification of some pathogens of veterinary importance.

## 5 Nanotechnology

An emergent topic in the development of new bioanalytical procedures, structures, and systems is nanotechnology, particularly for the generation of useful nanostructures for diagnostic applications; this is the so-called field of “nanobiotechnology”. Novel and improved electronic devices and biosensor platforms have emerged as a consequence of the inherent small size, enlarged surface area, and unusual optical, magnetic, catalytic, and mechanical properties of nanomaterials, unlike those of bulk materials [24]. Depending on their specific nature, for biosensing, nanomaterials may act as labels (including signal amplification), as biomolecule immobilization supports, or even as probes for specific biotarget anchoring. Certain nanomaterials can also be used for pre-concentration of biological targets. Among these applications, labeling has been the most commonly employed. Label-based detection methods are usually more time-consuming and labor intensive than label-free methods due to the labeling steps. Labels have also limited shelf lives and are subjected to leakage from sensing surfaces. However, label-based methods usually provide superior performance, especially in terms of sensitivity, than label-free ones. Moreover, standardized protocols with labeling procedures are already available. Fluorescence labeling has been, by far, the most common approach in this regard, although suffering from pH sensitivity and photobleaching over time [53]. Such handicaps and the advent of nano-engineering have propelled the search and development of new and improved labels.

Nanoparticles (NPs) have been the most widely employed type of nanomaterials for biosensing, especially metallic NPs. Metallic NPs are inorganic NPs that exhibit improved physicochemical characteristics compared to fluorescent labels, including higher sensitivity. In general, they are suitable for construction of high-density bioanalytical devices, taking advantage of their high signal-to-noise ratio (S/N). They are easily synthesizable and functionalized (by simple mixing at room temperature) and have a controlled, self-assembled surface structure [24]. Gold nanoparticles (GNPs), in particular, are already used frequently in molecular diagnosis; some of their advantages include low toxicity and versatility for many specific biorecognition applications and schemes. One common way to enhance the GNP signal even further, and thus the sensitivity of detection, is the inclusion of a final step of silver staining (“silver enhancement”), yielding detection schemes able to preclude the use of a prior PCR amplification step. The high sensitivity exhibited by many NP-based detection layouts, especially in the form of microarrays, has enabled to avoid a prior step of nucleic-acid amplification [69].

Quantum dots (QDs) constitute another class of metallic NPs, able for fluorescence tagging. They are much brighter and more

photostable than conventional organic fluorophores. Plus, their color can be directly correlated with size, while exhibiting very broad excitation wavelength windows, very narrow emission wavelength windows, and large Stokes shifts, allowing excitation at wavelengths far removed from their emission peaks [70]. Since QDs of different emission peaks (according to their different sizes) can be excited using a single wavelength excitation source, detection of multiple targets in complex biological systems is a hallmark of these NPs [71]. Another alternative to the overlapping of closely spaced fluorescence emission peaks and consequent limitation of the maximum number of fluorescent dyes that can be discriminated when simultaneously testing multiple pathogens in a single PCR tube is the use of masscode tagging, with a panel of distinct labels with different molecular weights. After an initial step of multiplexed (RT-)PCR using primers labeled with the masscode tags, unincorporated primers are removed, and the photo-cleavable tags of amplifying primers are then released by UV irradiation. Subsequent mass spectrometry analysis assigns each identified tag to its specific pathogen [1]. In principle, the multiplexing ability will only be limited by the highest primer concentration contained by a PCR mix. The method was applied to the identification of respiratory pathogens [72] and hemorrhagic viruses [73]. It offers a rapid, specific, sensitive, and cost-competitive alternative to conventional PCR and RT-PCR for disease diagnosis through POC devices. Nevertheless, some difficulties persist in miniaturizing mass spectrometer devices.

Among metallic NPs are also magnetic NPs (MNPs). Equivalent designations frequently found in the literature include “magnetic nanobeads”, “nanomagnets”, “nanomagnetic beads”, “nanomagnetic spheres”, and “nanospheres”. They have been vastly employed in many biosensor layouts for diagnosis. Despite not matching the nanosize of molecular recognition probes and targets, their microscaled counterparts, magnetic microparticles, are frequently preferred as magnetic labels for biosensing in view of the easiness for detecting the lesser abundant microbeads by routine optical microscopy or by magnetic detection and by the easiness of the purification process, thus allowing more efficient removal of nonspecifically bound labels, with enhancement of the assay performance [74]. However, the higher S/V of nanobeads provides much more binding sites for bioprobe and biotarget anchoring and hence a higher S/N [5]. Very often, magnetic particles are used for target pre-concentration from large initial sample volumes and purification, in parallel with the detection step itself being carried out through another particle that works as the label (e.g., fluorophore or GNP). In this case, there is an initial capture of the target by the probe-functionalized magnetic particle, followed by releasing of the target (“debinding”) for final detection. Through magnetically controlled removal of nonspecifically bound beads (magnetic washing), improved sensitivity can be

achieved upon elimination of the time-consuming washing step of nonspecifically bound molecules [75]. This process can, for example, improve significantly the detection specificity of genomic RNA, since RNA enrichment due to magnetic confinement also precludes the effect of common interfering substances and common RNA inhibitors [76]. In addition, the use of magnetic beads permits testing optically opaque samples [29], which is the case of many crude biological samples. Magnetic particles can be manipulated off-chip by a permanent magnet, making easier the design of disposable and inexpensive tests. Moreover, magnetic interactions are not affected by surface charges, pH, ionic strength, or temperature, being thus compatible with most biochemical processes [10].

Unlike inorganic NPs, organic NPs have enhanced structural flexibility and biocompatibility, while being biodegradable. Liposomes constitute an attractive type of organic NPs for efficient DNA-probe labeling and for signal amplification. This is commonly achieved by filling liposome particles with dye and fluorophore molecules, which amplify the response signal and are able to yield quantitative results. Another way of using liposomes in biosensing is in conjunction with resistive techniques. As such, negatively charged liposomes, upon binding to immobilized DNA chains (which are also negative), form giant negatively charged surfaces that repel the target DNA chain, leading to shifts in the electrochemical response [24].

In the last years, chemistry research has rendered a range of new structures based on carbon allotropes. The most promising for biosensing purposes seem to be carbon nanotubes (CNTs), both in the form of single- (SWCNT) or multi-walled CNTs (MWCNTs), depending on the number of cylindrical layers, with unique electronic properties and enlarged surface area for DNA immobilization. They also possess high electrical conductivity (similar to copper and much higher than in polymers), physical robustness, and chemical inertness. Each nanotube may act as an individual nanoelectrode, with sufficient free space between neighboring nanotubes preventing the overlap of their diffusion layers, therefore yielding high S/N values and hence improved detection limits [77]. By providing high sensitivity, they are amenable to PCR-free detection. Their production is sometimes unacceptably irreproducible for ultrasensitive detection, but this has been circumvented by using cheap CNT arrays for multiple biological targets as a way of averaging out between different batches [78].

The recent advances in nucleic-acid synthesis and modification processes and the discovery of nucleic acids with catalytic and regulatory activities have prompted the development of nanoengineered nucleic-acid analogues with new and improved abilities for biorecognition and diagnostic purposes. Among them are aptamers; they are synthetic nucleic acids able to interact with molecular or cellular targets with high specificity and sensitivity for their ability to fold into many tertiary conformations. Aptamers can be



generated by “Systematic Evolution of Ligands by Exponential Enrichment” (SELEX), a combinatorial procedure that starts with a pool of candidate nucleic-acid molecules to generate a nucleic-acid library [79]. Compared to antibodies, nucleic-acids can be synthesized in a more reproducible way, have longer shelf lives, and can be reversibly denatured without loss of activity. A remarkable characteristic of these probes for biosensing is that they do not require prior knowledge about the molecular differences between the specific target and nonspecific ones. As shown in cancer diagnosis, the DNA sequences from the DNA library that bind the cell-surface markers of a cancer cell can be determined by comparison with those that bind a healthy (control) cell. In addition, detection occurs before the corresponding antibody against that cancer has been produced [80]. This process is obviously attractive for application to the diagnosis of infectious diseases as well. The high selectivity and sensitivity achieved with aptamers permits eliminating sample pretreatment and is thus promising for POC applications [53]. The inability to distinguish the fluorescent signal from labeled and unlabeled probes is a common problem in microfluidic devices, since labeled probes that did not bind targets cannot be washed out from the microchannels. Different fluorescent labels can be used to tag the probe and the target, with the detection proceeding via fluorescence resonance energy transfer (FRET) upon the occurrence of the bioaffinity reaction. However, this procedure is unpractical in bioanalysis owing to the cumbersome dual labeling procedure [81]. In the case of DNA detection, this can be circumvented with the use of molecular beacons (MBs), which can be considered a particular type of aptamers. MBs are single-stranded oligonucleotides with a hairpin (stem-and-loop) structure, labeled with a fluorophore in one extremity of the chain and a fluorescence quencher in the other extremity. The close proximity between the extremities prevents fluorescence emission, but when a hybridization event occurs with a complimentary chain, the structure becomes linearized and hence fluorescence arises. In this way, target labeling is unnecessary. Another type of synthetic nucleic-acid analogues is constituted by peptide nucleic acids (PNAs), which are in which the sugar-phosphate backbone is replaced by a peptidic structure. When used as probes in nucleic-acid recognition systems, they allow very selective and sensitive hybridization in low ionic-strength media, while having high thermal stability [82]. For being electrically uncharged, PNAs are suitable to promote the occurrence of biochemical events triggered by the formation of the negatively charged PNA/single-stranded DNA hybrid, i.e., a kind of “on/off” processes.

Biosensing schemes reported in the literature employing at least one of the nanotechnology-based structures described above are depicted in Table 2, together with the transduction mechanism employed and performance quantification.



**Table 2****Illustrative works described in the literature employing nanostructures for the detection of animal pathogens**

Target pathogen(s)	Nanostructures <sup>a</sup>	Transduction mechanism	LOD or sensitivity	References
Canine parvovirus	PNA	Fluorescence	40–2,000 copies/ $\mu$ l (89.8 %)	[105]
Influenza virus (H5)	MB	Fluorescence	0.6 nM	[37]
Influenza virus (H5N1)	GNPs and Ag enhancer	Light scattering	$10^3$ TCID <sub>50</sub> units	[69]
Influenza virus (H5N1)	DNA aptamer	SPR	1.28 HAU	[87]
Influenza virus (H1N1)	GNPs	Fluorescence and surface-enhanced Raman scattering	–	[93]
Influenza virus (H5N1)	Complementary oxide semiconductor (CMOS)	Impedance spectroscopy	5 nM ( $10^{-11}$ F)	[98]
Influenza virus (H5N1)	DNA aptamer/hydrogel	QCM	0.0128 HAU	[102]
16 avian influenza viruses	Magnetic beads	Colorimetry (HA test and LAT test) and RT-PCR	16–1,024 HAU	[106]
Feline calicivirus	Liposomes	Fluorescence	$1.6 \times 10^5$ PFU/ml	[5]
Pestiviruses (Classical swine fever virus; Border disease virus; Bovine viral diarrhea virus 1 and 2)	Magnetic beads	Optic (visual; microscopy; chip reader)	–	[55]
<i>Alexandrium</i> sp. complex	PNA and cyanine-derived fluorophore (DiSC <sub>2</sub> (5))	Colorimetry	–	[89]
<i>B. anthracis</i>	SWCNT	Raman spectroscopy	–	[97]
<i>B. anthracis</i>	Electrically active magnetic NPs	Cyclic voltammetry	0.01 ng/ $\mu$ l	[94]
<i>B. anthracis</i>	GNPs	QCM	$3.5 \times 10^2$ CFU/ml	[95]
<i>B. anthracis</i> ; <i>S. enteritidis</i>	GNPs, magnetic NPs and NP tracers (PbS and CdS)	Square wave anodic stripping voltammetry	50 pg/ml	[90]

(continued)

**Table 2**  
(continued)

Target pathogen(s)	Nanostructures <sup>a</sup>	Transduction mechanism	LOD or sensitivity	References
<i>E. coli</i>	DNA aptamer	Impedance spectroscopy	10 <sup>-7</sup> M	[79]
<i>E. coli</i>	Alginate acid-coated Co magnetic beads	Transmission electron microscopy	10 cells/ml	[86]
<i>E. coli</i>	Fe <sub>2</sub> O <sub>3</sub> /Au magnetic NP and magnetic NPs	Amperometry	5 CFU/ml	[99]
<i>E. coli</i> O157:H7	Aluminum anodized oxide (AAO) nanopore membrane	Cyclic voltammetry and impedance spectroscopy	0.5 nM	[91]
<i>E. coli</i> O157:H7	Magnetic beads and QDs	Fluorescence	250 zM	[104]
<i>F. tularensis</i>	MB	Fluorescence	–	[84]
<i>M. avium</i>	GNPs	Colorimetry	1.875 ng/μl (87.5–100 %)	[100]
<i>M. tuberculosis</i> , <i>M. bovis</i>	GNPs	Colorimetry	5 × 10 <sup>-8</sup> M	[85]
<i>S. aureus</i>	GNPs/poly-3,4-ethylenedioxythiophene (PEDOT) film	Chronoamperometry	≤150 pM	[96]
<i>S. aureus</i>	GNPs/PANI nanofibers	Cyclic voltammetry	pM range	[101]
<i>S. aureus</i> (MRSA)	PNA	Impedance spectroscopy	10 pM	[103]
<i>Y. enterocolitica</i>	Carbon ionic liquid electrode and V <sub>2</sub> O <sub>5</sub> nanobelt/MWCNT/chitosan	Differential pulse voltammetry	1.76 × 10 <sup>-12</sup> M	[92]
<i>C. perfringens</i> , <i>C. tetani</i> , <i>S. pneumoniae</i> , <i>P. aeruginosa</i> , <i>E. coli</i>	GNPs	QCM	1.5 × 10 <sup>2</sup> CFU/ml (94.12 %)	[83]
Salmonellae	GNPs and Ag enhancer	Colorimetry	10 <sup>4</sup> cells	[88]

<sup>a</sup>Micro-scaled magnetic particle labels are also considered in this table

## 6 Conclusions

The advances observed on the past decades in proteomics and genomics have led to the discovery of novel diagnostic biomarkers for pathogens relevant in human medicine. In parallel, cutting-edge developments in materials science and in nanotechnology

have also been registered. Veterinary science and practice “took the train” and, as such, have been greatly favored from such advances. Microfluidic technologies and nanoengineered structures, especially when coupled together, have led to an unprecedented degree of high-throughput, large-scale genetic analysis, even at whole-genome levels. Ultimately, the high-throughput and multiplexing abilities of *in vivo* (implanted or swallowed) nanosensor arrays should be able to monitor animals’ physiology and health status during their entire lifetime, and even beyond, intended to track and assess the quality of animal products for human feeding. In this way, the current shortcomings related with the limited number of sanitary surveillance resources that can be allocated to guarantee proper product origin, stocking, and shipping could be circumvented. So far, only few POC schemes and devices have reached the exquisite sensitivity thresholds required for detection of nucleic-acid traces in unamplified biological samples, which is the ultimate goal of nucleic-acid testing. As for human diagnostics, the veterinary medicine and practice still lack the commercial availability of more POC devices, more probably as a result of manufacturing and commercial cost-effectiveness constraints than to a shortage of fundamental knowledge. While remaining too expensive for single testing, many tests targeted for POC diagnosis will ultimately prove to be cost-effective when savings with unnecessary laboratory manpower are taken into account. Other major challenges are the need for initial investments that are often prohibitive for small companies and the usual difficulties for obtaining regulatory approvals for testing and commercialization. For effective improvement of human health, more adequate and coordinated actions to face animal diseases are needed, especially concerning livestock. This includes a more effective technology transfer from developed countries to those where diseases are prevalent and where disease preventive measures may be crucial to avoid or contain epidemics. Probably still without meritorious examples in the world, more effective communication and coordination among public health, animal health, and wildlife disease surveillance authorities will be necessary to tackle the problems posed by common and hazardous veterinary diseases, especially in situations of outbreaks endangering animal and human health as well.

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