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Review Article

Molecular biological tools applied for identification of mastitis causing pathogens

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ABSTRACT

The molecular diagnostic tools became the gold standard of mastitis diagnosis in the last few years. They enable rapid, qualitative, quantitative and large scale diagnosis. In addition to their role in diagnosis, they can identify pathogens at the subspecies level which is necessary for the epidemiological studies. They are increasingly used in mastitis control programs through identification of suitable candidates for vaccine production and through the selection of mastitis resistant cattle breeds. The present molecular techniques are continuously improved and new techniques are developed in order to provide higher sensitivity and specificity and to minimize the costs. The present work aims to provide an overview of the modern molecular tools, discuss why they replaced the traditional tools and became the new gold standard in mastitis diagnosis through comparing both traditional and molecular tools, explore the prospective of the molecular diagnostic techniques in mastitis diagnosis and control and to explore new horizons of using molecular assays in near future.

Contents

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1. Introduction

Mastitis is the most costly problem in dairy industry. The resulting economic losses include direct losses as (1) temporary/permanent decrease in milk production, (2) reduction of milk grade in subclinical cases due to the increased number of somatic cells and (3) the fully rejection of milk in clinical mastitic cases or due to antibiotic residues. In addition, indirect losses are also included as (1) premature culling of dairy cows and early replacement cost, (2) low cow sale price, (3) additional veterinarian and medication costs, and finally (4) the diagnostics/laboratory expenses [1,2].

Mastitis is the inflammation of the udder tissue which could be prompted by various infectious agents. It is characterized by the elevation of somatic cell count, and usually accompanied with physical, chemical and/or microbial changes of the milk. Mastitis pathogens are usually bacteria, however, molds, yeast, and prototheca may also induce mastitis. It is important to identify the mastitis prompting organism in order to (1) properly treat and select the suitable antibiotics, (2) understand their route of spread and evaluate the contagiousness of the case, (3) calculate their public health impact, (4) to judge the prognosis of the affected quarter/cow considering early culling decision, (5) select the suitable hygienic and preventive measures, and finally for (6) choosing the proper mastitis vaccine programs to be applied in the farm [2,3].

Field diagnosis of clinical mastitis is usually based on udder examination, changes in the physical properties of the milk and the increase in somatic cell count and even the use of ultrasonography [3,4] while the diagnosis of subclinical mastitis is more difficult and depends on indirect techniques such as California Mastitis Test (CMT), electrical conductivity, or the detection of body enzymes released due to tissue damage (e.g. LDH, and NAGase) [2].

An ideal diagnostic test must be sensitive, specific, rapid, repeatable and economic. Most conservative laboratories worldwide still consider bacterial isolation and cultivation to be the (Gold Standard) for the diagnosis of mastitis. The question if the culture still the gold standard and whether the PCR replaced it and became the modern gold standard is debated because both techniques have their strength aspects and weakness points (Table 1; [5,6]).

2. Major mastitis causing pathogens

More than 150 different bacterial species and subspecies are involved in the induction of bovine mastitis [7], out of these; only 10 groups are responsible for 95% of the recorded cases worldwide [2]. These pathogens classified as environmental or contagious pathogens depending on their primary reservoir and mode of

Table 1
Comparison between the old and the new gold standards: Bacteriological assays versus PCR showing the strength and weakness points of both techniques.

	PCR	Culture	Refs.
Technicality and costs	It is more expensive and requires special infrastructure and well trained skillful persons.	Standard media used for primary screening are always available in most laboratories. However, they are not suitable for isolation of some pathogens such as Mycoplasma or *Mycobacterium bovis*.	[2,14,50]
Bias	PCR inhibitors present in mastitic milk, improper extraction or purification of the DNA from the sample may lead to false negative results. The use of column purification is recommended, however, if the mastitic milk is clotted, the purification process may be inefficient. The use of internal controls can differentiate between truly - and false negative results. False positive results can occur due to nonspecific amplification if less restrictive PCR conditions are applied or if the primer selected is not specific enough. False positives due to DNA carryover effect and from contamination or teat canal colonization may also occur.	About 30% of milk samples taken from clinical and subclinical cases revealed negative bacterial growth after 48 h of incubation due to the death of the causative agent during transport/sample storage, the use of unsuitable culturing media or due to the presence of antibiotic residue or preservatives in the sample which inhibit the bacterial growth but not their molecular detection. Also due to overgrowth of contaminant microbes during sample transportation which may mask the real mastitis inducing microbes.	[5,32,73–76]
Public hazards	The use of Ethidium Bromide is a serious source of environmental contamination and public hazards.	The enrichment of the pathogens may lead to biological contamination/public health hazards can lead to laboratories acquired infections.	[77]
Screening capacity	Faster and adapted for screening purposes with lower costs per detected agent. The PCR can only detect the target pathogens according to the used However, the use of multiplex PCR overcomes this disadvantage	Has a broad spectrum screening capacity if the milk sample is cultured on blood agar a 37 °C for 48 h. Time consuming (24–48 h) and laborious especially if slow-growing bacteria are suspected.	[78]
Sensitivity and specificity	Higher sensitivity and specificity values due to its ability to detect both viable and killed organisms. PCR usually requires a small amount of target DNA and therefore has a higher detection limit.	The culturing process is not easily inhibited compared to PCR which can be inhibited by a wide range of PCR inhibitors present in mastitic milk such as proteinases, calcium ions, lactoferrin (leukocytes) and heme (in bloody milk), or due to programing mistakes of the thermocyclers.	[79,80]
Accuracy and repeatability	High detection level. The RT-PCR enables pathogen quantitation.	Culture enables multiplication of pathogens if present at low concentration and reflects the true active intramammary infections unlike PCR, because it detects only viable bacterial cells.	[29,30]
Typing	Differentiates among different genotypes of the same species and deliver antibiotic resistance profiles, which enables rapid treatment of mastitic cows.	Serotyping is not efficient enough. The antibiotic resistance test can be done but it is laborious and time consuming.	[8]
Others	The results delivered by PCR are in the form of digital data, which can be easily exchanged or stored. It is easier to store the PCR product (in refrigerator or freezer) for long periods, than storing cultured petri dishes, which will dry or will be masked by fungal growth if not properly preserved. Lyophilization of the culture or freezing in glycerin may offer good solutions for culture storage.	The source of the material (the grown cultures) remains available for test repetition/confirmation or for further investigations if needed in opposite to PCR when applied directly on the sample not on a culture. The source of the investigated material can multiply (as subculture) if the material was nearly exhausted or needed in large amounts, in opposite to extracted DNA in case of PCR.	[32,75,81,82]

transmission. The most common contagious pathogens are *Staph. aureus* and *Mycoplasma* spp. while the environmental group is represented mainly by *Strept. uberis, Strept. dysgalactiae*, coliform bacteria and other Gram negative bacteria such as *E. coli, Klebsiella pneumoniae, Serratia, Pseudomonas* species, *Proteus* species, and *Pasteurella* species. If high coliform/Gram-negative bacterial count is recorded in mastitic milk, it might be associated with mastitis, but most probably it is related to bad hygiene such as improper cleaning of the milking system, improper milking procedures, or inadequate cooling of the milk, or other environmental contamination sources. In the same way, the presence of *Staph. aureus* in the sample may indicate infection of the udder with *Staph. aureus,* or milk contamination from the teat skin, hand/wounds of the workers or the surrounding environment [8]. Additional microbes are less commonly known to be mastitis inducers; such as *Arcanobacterium pyogenes*, other non-aureus *Staphylococci*, *Corynebacterium bovis, Mycobacterium bovis, Strept. parauberis, Strept. Agalactiae, Strept. zooepidemicus, Strept. equinus, Strept. canis, Enterococcus* species including *E. durans, E. faecalis, E. faecium,* and *E. saccharoluticus, Bacillus* species and other Gram-positive *bacilli- Bacillus cereus* and *B. subtilis, Nocardia species, Prototheca,* Yeast [2,4,9,10].

However, this traditional classification of the mastitis inducing microbes into environmental and contagious microbes started to be modified through recent data delivered by the application of modern molecular epidemiological tools which counteracted the old classification. The data showed that within the same species some isolates can be classified as contagious and others as environmental, some as extremely pathogenic and others less pathogenic, some cause severe clinical mastitis others mild subclinical mastitis. This, in turn, offers a huge bonus point for molecular genotyping based assays compared with the conventional bacteriological tools [11–16].

3. Molecular biological methods used for mastitis diagnosis

Various DNA-based identification assays can be used for the characterization of pathogens at different phylogenetic levels according to the aim of the test and primer design. These methods can detect either DNA or RNA. While the extraction or detection of DNA is more common and often technically easier than that of RNA due to the higher stability of DNA than RNA. For this reason, the DNA-based detection assays can detect non-viable and/or inactivated pathogens in opposite to those assays targeting the mRNA which is less stable and therefore can detect only viable pathogen. On the other hand, the detection of the genes encoding antibiotic resistance does not necessary mean that the bacteria are resistant against antibiotics, but the detection of mRNA resulting from gene expression will deliver more accurate results [17,18].

4. Molecular markers of infectious mastitis inducers: Identification and genotyping

A DNA signature means the identification of unique DNA sequences in the genome of a particular organism, which is absent in all other, even the closely related, microbes (Table 2). These Phylogenetic markers help in bacterial characterization such as the *16S rRNA* or *23S rRNA* genes. Such highly conserved genetic sequences are usually the first choice for primer design. In opposite to those highly conserved markers, it is not common to depend on virulence genes alone for bacterial identification as they are highly dynamic among related bacterial species/subspecies due to their location on mobile genetic elements which can even be transmitted from one species to another. However, if the planned reaction aims to differentiate among different genotypes of the invading microbe, certain genomic hotspots, polymorphic sequences, intergenic spacers and accessory/virulent genes can be selected for this purpose [14].

5. Preliminary step: DNA extraction

The concept of the molecular biological techniques is based on primarily extraction of template DNA from the samples with parallel elimination of available reaction inhibitors such as somatic cells. For this purpose, new methods are developed to increase the concentration and purity of the DNA and decrease the cost of purification, ranging from culture boiling to the use of commercial DNA extraction kits, lysis buffer, magnetic beads, or the use of Silica column. Pre-PCR enzymatic treatment of the bacteria leads to a clear improvement in the detection level of the PCR specially if the suspected pathogen is a gram positive organism. The cost of the DNA extraction per sample varies according to the used method, ranging from 0.16 to 4.30 USD [9,19–24].

5.1. Polymerase Chain Reaction (PCR)

PCR is an in vitro amplification of unique organism specific target DNA sequences using sequence specific oligonucleotide primers and heat stable polymerase. The selected primers must have an exclusive sequence that bind specifically and selectively to previously defined DNA target sequence. The primers may either be designed to differentiate among members of the same species or to identify the organisms at subspecies level. By so doing, the primers allow the amplification and quantification of certain sequences. For the diagnosis of present pathogens, the primer target sequence must be highly conserved within all strains of the suspected species to avoid false negative results but variable among other species to avoid cross reaction resulting in nonspecific annealing leading to false positive results [2,8,15].

Different PCR systems were developed to offer a rapid, accurate and economic diagnosis of causative agents of mastitis. PCR can be applied on quarter -, pooled - and bulk milk samples. The sensitivity of detection limit decreases with the more dilution (pooling/mixing) of infected milk with healthy milk. The quarter milk samples deliver the most accurate data about the predominant pathogen in the farm with a clear higher level of sensitivity and specificity in comparison to pooled or bulk milk samples. On the other hand, the application of modern molecular tools in investigating pooled or bulk milk samples can deliver accurate data comparable with that data delivered when using quarter samples. PCR

Table 2
Genetic markers for the identification and typing of mastitis inducing pathogens.

Mastitis inducer	Genetic markers for diagnosis		Refs.
	Diagnosis	typing	
Staph. aureus	*nuc* or *16S rRNA*	*coa, aroA, clf, cna* and the *spa* gene and its Xr-region.	[15]
CNS	16S *rRNA*, *tuf*, and *rpoB*	RFLP on *gapC* gene, PCR for tRNA intergenic spacer, AFLP, and (GTG) 5-PCR typing.	[83]
Streptococci	*cpn60*	*cpn60*	[14,27,28,84]
Strept. uberis	*sod*A, *cpn60*	*yqiL*., *hasA*,	[27,85]
E. coli	16S rRNA	enterobacterial repetitive intergenic consensus (ERIC).	[86]
Mycoplasma	*16S–23S rRNA p40* -	MLST of *fusA, gyrB, lepA* and *rpoB*	[25,87,88]

can detect one moderate to heavily infected cow with *Strep. agalactiae* or *M. bovis* even if the milk was pooled with milk samples from 1000 healthy cows in the herd [25]. Testing multiple samples collected over time enables efficient and accurate herd screening against all mastitis inducing pathogens even those which are intermittently shed such as mycoplasma [2].

5.2. Multiplex PCR

Multiplex PCR is a PCR system, in which many primer pairs are mixed together in the same PCR reaction. The used primers can either target genes belonging to different pathogens for screening (diagnostic) purposes or target different genes within the same pathogen for genotyping purposes. Different sets were developed for commercial purposes so that the identification of the mastitis pathogens became easy, rapid and standardized [9,26].

Compared to conventional PCR, the Multiplex PCR is cost effective and more practical in routine diagnostic institutions. The multiplex PCR reduces the cost per sample and the time required for sample screening for the presence of different pathogens, or to search the presence of different virulence genes within the same isolate. It is usually 10–100 folds less sensitive than the simplex PCR using the same primer pairs separately due to the competition of the primers for nucleotides and other reagents or increase the possibility of primer annealing together [26–28].

Shome and his team [7] developed a Multiplex PCR which can simultaneously detect 10 major mastitis inducing bacteria, the developed assay was shown to be more efficient than bacterial culture in mastitis diagnosis and can detect 10 fg of bacterial DNA and $<10^3$ CFU ml^{-1}. It is advisable to randomly culture mastitic samples beside the routine PCR application in order to roll out accidentally/rare mastitis inducers as the established PCR sets are designed for the detection of the most common inducers [2].

5.3. Real-Time PCR (RT-PCR)

The use of RT-PCR offers additional benefits compared to the bacterial culture and conventional PCR. It is not only faster and more sensitive, but also more safe for the workers and environment (no ethidium bromide is used), post reaction handling is not needed (no agarose electrophoresis), better visualization and digitization of the results which enables documentation and data exchange with other teams. The sensitivity and specificity of RT-PCR in the diagnosis of mastitis pathogens may reach 100%. In addition, RT-PCR can be applied for the quantitation of pathogens in the infected milk through measuring the intensity of the produced fluorescence from the reaction [29,30].

The results can be obtained within 4 h after the arrival of the samples to the laboratory compared to several days for the culture. The rapid and accurate diagnosis of the causative organism is of a great value to start the therapy as soon as possible [14]. Commercial kits are available for the simultaneous detection of the major mastitis pathogens as *Staph. aureus*, *Strept. agalactiae* and *Strept. uberis* directly from mastitic milk using a multiplex RT-PCR assay with an accuracy of 98% [31].

Although RT-PCR offers a quantitative analysis of the bacterial load in the sample, this application still faces some limitations in the repeatability of test result and difficulties in the interpretation of the results of some commercial multiplex RT-PCR assays. This is attributed to the difference in the quality, concentration and purity of the extracted DNA from the sample according to the applied DNA extraction method or kit [23].

The concept of RT-PCR is based on the usage of different types of fluorogenic DNA probes such as TaqMan and molecular beacons (MBs). The major difference between both dyes is that the TaqMan, in opposite to MBs, build no "hairpin" arrangement. It exploits the 5′-3′ nucleolytic activity of used polymerase in order to split the fluorescein from the 5′ end of the probe which was hybridized downstream from forward primer in the running reaction. This step is followed by the separation of the fluorescein from the quencher so that the fluorescence emission starts. The used 5′ end reporters for TaqMan include the 6-carboxyfluorescein (FAM), the tetra chloro-6-carboxyfluorescein, and the hexachloro-6-carboxyfluores cein, with the quencher dye 6-carboxytetramethylrhodamine (TAMRA) which attaches almost two bases downstream from the reporter. The closeness of both reporter and quencher decreases the degree of the reporter emission intensity until the reporter gets splitted [32]. On the other hand, the MBs have "hair-pin" structure start to fluoresce when being hybridized with their complimentary sequences. MB probes are clearly more sensitive than TaqMan, so that the MB based PCR sets can detect only two colony forming units in the sample. In general, if low concentration of bacterial DNA is present in the sample, it will need more time and more cycles to exaggerate enough to produce detectable fluorescence levels. The probes are made of stem structure with two complementary arms on both sides. One two arm is attached to fluorescent moiety while the second is attached to quenching moiety. The quenching effect prevents the fluorescence as long as these two arms are close together. The fluorescence can only occur when these two arms are separated, i.e. when the probe hybridizes with the complementary target sequence in the template DNA [33]. In conclusion, the samples are considered to be positive when the degree of fluorescence emitted from the reaction exceeds the preset threshold level. The lower the concentration of the microbial DNA in the sample, the more cycles it needs to produce enough fluorescence to be detected by the system. Usually, the samples are considered to be negative if there was no detectable fluorescence after 40 cycles. If this threshold is reduced, the test specificity will be increased while its sensitivity will be reduced. Monitoring of fluorescence after each annealing stage enables the monitoring of the progress of the reaction. The cycle threshold (Ct), is defined as the cycle at which a significant increase in fluorescence eminences can be detected, and is helpful to estimate the initial concentration of the template DNA in the sample. Multiplex Real Time PCR can also be used for rapid diagnosis of mastitis inducing pathogens. In such reactions, different dyes are used where each target sequence is visualized by his own color, therefore, little number of target sequences can be searched per reaction in opposite the conventional Multiplex PCR [2,33,34].

6. Other variants of PCR

Different variants of PCR were developed in the last few years. However, they are all based on the same concept. Triplex PCR is a simplified modification of Multiplex PCR, in which only 3 oligonucleotide primers are used. This variant of PCR enables us to differentiate between two suspected closely related organisms. The primer design is based on the selection of one shared target sequence and two reverse unique sequences for the two organisms. In the same way, it is also possible to design a more complex form in which variable number of forward primers is added to face less number of reverse primers in the reaction. This reduction in the number of primers in the reactions minimizes possible faulty annealing or dimer building [3]. Another variant is the combination of different assays together such as performing Multiple PCR followed by electrospray-ionization mass spectrometry (PCR/ESI-MS) in order to enable rapid detection of nearly all known pathogens. This technique is based on DNA amplification by PCR targeting specific sequences of ribosomal, other highly conserved house-keeping genes and certain antibiotic-resistant genes. This is then followed by their spraying into a time-of-flight mass spectrometry for the

determination of their molecular weight. The mass of each amplicon is then translated into base composition for the identification of the pathogen and the harbored antibiotic resistance genes [3,35].

7. PCR-ELISA

Polymerase chain reaction-based enzyme linked immunosorbent assay (PCR-ELISA), also called PCR-enzyme-linked oligosorbent assay (PCR-ELOSA), is a technique resulting from the combination/mixing of both PCR (molecular technique) and ELISA (serological technique). The used ELISA is adapted to detect biotinylated nucleic acid rather than proteins in order to sero-/genotype pathogens, enables large scale screening of samples or for quantitative analysis of PCR products. The use of PCR –ELISA increases the sensitivity and specificity of the pathogen detection in milk with shorter analytical time as reported by Daly and colleges [36] who could detect only 5 CFU of *E. coli* in the milk. The key for the increase in the sensitivity is the fact that the efficiency of visualization using an enzymatic colorimetric assay of the hybridized biotin labelled probe with PCR amplicons is much better than that achieved through ethidium bromide stained agarose gel electrophoresis. The incidence of false positive results due to DNA contamination is clearly less than PCR due to the use of DNA probes followed by amplicon hybridization. In comparison to PCR, the PCR ELISA is not only more sensitive and specific but also safer and friendlier for the environment due to avoidance of the mutagens ethidium bromide dye. In addition, the PCR ELISA is considered to be a semi-quantitative tool as the concentration of the target specific DNA is correlated to the intensity of the obtained color. These advantage can makes the PCR ELISA comparable with RT-PCR, however the RT-PCR is more trustable as quantitative technique but can only be performed in good equipped labs with advanced infrastructure [36,37].

The new techniques can be applied to diagnose mastitis inducing pathogens specially mycoplasma which are the smallest known bacteria. They are not affected by many antibiotics as they lack the cell wall. There are many methods which are used for the diagnosis of Mycoplasmosis such as culture, FAT, and PCR, while the serological techniques detect only infections older than 14 days. Recently developed biosensors can detect Mycoplasma infection rapidly, easily and with a high specificity level. Most of the biosensors are based on the detection of NAGase and haptoglobin markers in mastitic milk samples. The detection of both bacterial proteins indicates the presence of acute inflammation; however, false positive results may be obtained if the milk sample contains traces of blood. On the other hand, the ideal biomarker for the diagnosis of *Mycoplasma bovis* is the surface protein called P48 protein. The use of a biotin-labeled single-stranded DNA aptamer specific for P48 protein of *Mycoplasma bovis* was applied in a competitive enzyme-linked aptamer assay for the diagnosis of *mycoplasma bovis* infection in serum samples with a very high degree of sensitivity and selectivity [38].

8. Micro array

The use of Microarray assay is a technically demanding and costly technology which is usually applied in research laboratories. The microarray technology is based on the hybridization of hundreds of target genes loaded on the microarray chips followed by their visualization through being exposed to sequence complimentary DNA probe conjugated with fluorescence or chemiluminescent stains [39]. The arrays are slides on which robotically added DNA is spotted to permit genome analysis of the investigated pathogens. The technique is carried out in four stages: (1) firstly, DNA extraction, (2) followed by PCR of target genes, (3) DNA hybridization, and finally (4) reaction visualization through colorimetric methods. i.e. a mastitic milk sample will be first subjected to DNA extraction process (to release the microbial DNA), followed by PCR targeting specific DNA fragments or genes. The PCR products are then transferred on the chip, on which DNA probes (complementary to the amplified genes) are pre-spotted. After hybridization, washing of unbound DNA ensures the specificity of the colorimetric signals. This combination of techniques enables powerful identification through, high screening capacity of hybridization and increased sensitivity, specificity and efficiency of the colorimetric visualization [14,39]. Modern microarray systems enable protocol automatization and the development of multiplex biochips for identification of up to seven different mastitis causing pathogens in one reaction with a detection limit of 10 3 CFU/mL milk [40].

Lee and his team [39] developed a biochip which can detect the most common species of mastitis-inducing pathogens, namely; *Corynebacterium bovis, Mycoplasma bovis, Staphylococcus aureus,* and the *Streptococcus* spp. *Strep. agalactiae, Strep. bovis, Strep. dysgalactiae,* and *Strep. uberis*, within few hours. More recently, a combination of PCR and a nucleic acid microarray immunoassay was developed. This technique enables colorimetric identification of 6 field strains from 4 different pathogens in one milk sample in less than 3 h [41].

9. Hybridization/blotting

They are relatively old techniques which are demanding, costly and time consuming. They are usually used for screening purposes. There are different blotting techniques which share the same fluorescence concept. The oldest blotting assay is the Southern blotting, which was named according to its developer Edwin Southern. It depends on the separation of DNA fragments on agarose gel by gel electrophoresis for the detection of a target DNA sequence [42,43]. In opposite to Southern blot, the Western blotting is applied on separated protein bands (instead of DNA bands) with the aid of monoclonal antibodies. A combination of both techniques can be used to find the correlations among proteins and DNA fragment and to find out which genes are responsible for the production of certain proteins. Meanwhile, the Northern blotting targets denaturated RNA sequences to investigate gene expression. Unlike PCR, the hybridization concept depends on the use of a single stranded fluorophore labeled DNA probe, which is much longer than the oligonucleotide primers used in PCR. By so doing, it is delivers highly specific results if done under high stringency conditions. If different probes are labeled with different fluorophores colors in the same reaction, the assay can be used to simultaneously detect different target genes or pathogens [42,43].

In most cases, hybridization requires a preparatory step, in which a PCR amplification of the DNA probe is needed. Dot blot hybridization is a more practical and economic alternative technique for the microarray, in which the microarray slide is replaced by a nylon membrane to be loaded with either the DNA of the investigated pathogen (in case of traditional dot blot) or loaded with multiple DNA markers (in case of inverted dot blot), to enable simultaneous detection of different selected markers [14,43].

Recently, the *in situ* hybridisation (FISH) technique was used to detect mastitis inducing bacteria. The assay can be adapted to simultaneously detect different pathogens if present at concentration $\geq 10^6$ CFU/mL. The used DNA probe has to be labeled before hybridization. This can be achieved by nick translation, random primed labeling, or PCR. The labeling can be performed either through direct (using fluorophore) or indirect (using hapten) labeling technique. When using indirect labelling with nonfluorinated haptens, enzymatic or immunological detection assays are required for the visualization of the reaction [43,44].

At the time, there are many newly developed techniques which are based on the hybridization of nucleic acids and are usually used as rapid and economic screening tests. There are various commercial kits as the GenTrack assay which uses a dip stick format. The techniques target usually the rRNA to increase the test sensitivity due to the presence of huge copy numbers (many thousands) of rRNA in every bacterial cell. However, the specificity of such tests is usually low due to the close relationship of rRNA sequences among related bacterial species [45]. The use of Blotting assays faces many limitations in the field as they are time consuming, labor-intensive and less sensitive, less quantitative when compared with modern equivalent techniques such as RT-PCR. The degree of specificity and sensitivity can be controlled according to the reaction conditions and the used reagents [42].

10. Use of molecular tools in identification of mastitis causing pathogens at subspecies level

Some may ask why do we need to distinguish among related strains and to recognize the mastitis causing genotype. Actually, these data have great value for epidemiological studies aiming to track the source/focus of infection, identification of transmission routes, determine the contagiousness of the isolates, and evolution of new virulent strains and their antibiotic resistance profile. Additionally, they used for development of efficient vaccines leading to the improvement of mastitis control program in the region. The present work focused on *Staph. aureus* genotyping as an example because the S*taph. aureus* genome is well studied due to its potent role as mastitis inducer and because of its zoonotic importance. The identification of the present genotype is important as only a limited number of *Staph. aureus* field isolates play a major role in the induction of clinical mastitis, while other related strains exist normally on the teat skin and may accidentally contaminate the milk samples. Due to all these reasons, great attention is given to the genotyping of *Staph. aureus* field strains [8,46,47].

Pathogen typing can be performed by phenotypic or molecular typing techniques. Phenotypic characterization techniques like serotyping, biotyping, phage typing, or the multilocus enzyme electrophoresis technique mostly have a week discriminatory power and many field isolates are reported to be untypable [17,47,48]. Usually, the phenotypic tests are based on the detection of certain differences in bacteria metabolism. Therefore, the application of these assays is restricted to living cultivable organisms. They have a low accuracy level [49,50]. For genotyping purposes, primers targeting accessory genes as molecular markers are usually designed e.g. virulence genes of *Staph. aureus* needed for the establishment of primary adhesion or invasion of the udder tissue. The available genotypic methods are faster (e.g. RT-PCR) and more discriminatory (e.g. Randomly Amplified Polymorphic DNA/RADP methods [32].

The differentiation of the isolates at the subspecies level is also important to distinguish bovine isolates from human isolates. The ß hemolysin gene (*hlb*) was found to be more associated with *Staph. aureus* isolates of bovine origin and increases both the damaging effect alpha hemolysin and the adhesion capacity of *Staph. aureus* to the mammary epithelium [8,15,51]. As the ß hemolysin gene (*hlb*) is not necessary for human invasion, the present β. hemolysin encoding genes in the bovine isolates mostly become distrusted (phage conversion) by the prophage (phiNM1-4) encoding different virulence genes in addition to the modulators of innate immune responses (*sea, sak, chp* and *scn*) when being adapted to humans [8,15,51]. In a very interesting older investigation, the research team subcultured phenotypically β. hemolysin negative *Staph. aureus* isolates of human origin in bovine blood and bovine milk. About 90% of the phenotypically negative isolates recovered their capability to produce the β. hemolysin after being subcultured for 1–4 times in cow milk or 5–10 times in cow blood. On the other hand, no changes were noticed in the expression profile of *Staph. aureus* isolates of bovine origin when cultured in human milk or blood [52]. This phenomenon can be explained as the repeated passage of the *Staph. aureus* isolates in bovine milk or blood lead to the loss of the prophage disturbing the β. hemolysin gene sequence, leading the resume of their functionality and the ability to express the gene again [53–56]. Recent data showed that although the staphylococcal superantigen enterotoxins are usually incriminated in the induction of food poisoning, *Staph. aureus* isolates that produce the Enterotoxin D (SED) alone or in combination with Enterotoxin C (SEC) and the Toxic shock syndrome toxin (TSST) are capable of induction of sever grade of usually incurable mastitis [57]. It was also noticed that *Staph. aureus* strains that produce the enterotoxins SED and SEJ are linked to persistent mastitis and mostly co-exist with the penicillin resistance gene *blaZ* [51]. Taking *Staph. aureus* clone CC8 as an example, the dynamic conversion of human type *Staph. aureus* CC8 to bovine type CC8 was done through the combined loss of both β. hemolysin converting bacteriophage and the acquisition of a non-mec staphylococcal cassette chromosome (SCC) from the animal environment. This SCC encodes a LPXTG-protein which could play a role in bovine colonization [58]. The *Staph. aureus* isolates can express more than 20 types of Adhesines, most of them are responsible for the primary adhesion to udder tissue to establish invasion such as the clumping factors A and B encoding genes (*clfA, clfB*), the collagen adhesion encoding gene (*cna*), and the fibronectin binding protein A and B encoding genes (*fnb*A-B) [59].

Due to the zoonotic importance of certain *Strep. agalactiae* or group B streptococci, it is necessary to differentiate among human and bovine strains. This can be achieved for example through the application of 15-gene MLST assay which clusters the bovine strains apart from the human ones. Isolate differentiation based on other genetic markers is also common in diagnostic labs, such as the detection of the C5a peptidase gene *spcB* and the laminin binding gene *lmb* which are characteristic for human field strains and only present in about 20% of bovine isolates. Additional markers as *infB*, *sodA* and *gdh* alleles can also be used for the same purpose. On the other hand, some genomic islands are more common in *Strept. agalactiae* strains of bovine origin such as the lactose utilization operon. The presence of these operons is reflected (phenotypically) on their ability to utilize lactose which enables them to survive inside the udder, therefore, is present in 92% of bovine compared with only 13% of human *Strept. agalactiae* isolates. Due to its role in the mastitis pathogenesis, this operon is also shared in mastitis inducing *Strept. dysgalactiae* field isolates [60].

Many protocols are used to type *Strept. uberis* such as the double MLST scheme. While the first MLST scheme (Multilocus Sequence Typing) covers many housekeeping genes, virulence genes and vaccine targets, the second scheme concentrates only on housekeeping genes. Certain genes became more common in use such as the housekeeping gene *yqiL*. A common genetic marker is the capsule gene *hasA*, which is strongly associated with mastitis prompting isolates. Although *hasA* is not necessary by itself for the pathogenesis of mastitis, and even the strains subjected to *hasA* deletion mutation were also capable of inducing bovine mastitis, it is usually associated with bovine mastitis inducing strains. This may be attributed to the coexistence of this gene with other virulence genes, which are needed for the induction of bovine mastitis, on the same mobile genetic element [12,60].

Strain typing of *E. coli* is usually based on the enterobacterial repetitive intergenic consensus (ERIC). The use of PFGE assay enables the differentiation between mastitis inducing *E. coli* strains and environmental isolates. Different PCR sets are now available for molecular diagnosis of *M. bovis* in milk. The use of primers tar-

geting the *16S rRNA* gene sequence of *M. bovis* offers a rapid, accurate and reliable alternative [12,60].

The most common molecular typing concepts are based on sequence polymorphism or size polymorphism. The size polymorphism dependent techniques are based on electrophoretic separation of amplified DNA fragments of variable molecular lengths on agarose gel [8,32]. Molecular marker technology targets hot spots in the genomes or genomic sites showing sequence mutation, insertion, duplication, inversion. Certain abbreviations are commonly used such as the SNP (Single-nucleotide polymorphism) describes the presence of a sequence polymorphism resulting from a single nucleotide mutation at certain genomic loci, the SSCP (Single-strand conformation polymorphism) delivers rapid information about sequence differences in closely related pathogens in an economic manner, VNTRs for (Variable Number of Tandem Repeats) and SSR (Simple Sequence Repeats/microsatellites) [61].

Meanwhile, the detection of sequence polymorphism can be achieved through full or partial genome sequencing which is costly and is not applicable in most routine diagnostic Laboratories. Sequencing of RNA or DNA yields clear data, easy to be compared, interpreted and exchanged. A cheaper alternative to sequencing for the detection of sequence polymorphism in bacterial genome is the use of restriction enzymes [32].

Terms like "genotyping, fingerprinting, or molecular subtyping" are used to refer to strain/pathogen DNA based typing which aims to further characterization of the isolated pathogen beyond the species or subspecies level. Different techniques were developed for this purpose such as (1) Pulse field Gel Electrophoresis Technique (PFGE) which depends of the separation of enzymatically digested DNA bands by gel electrophoresis. It is a labor-intensive, expensive and time-consuming technique. In addition, it can be carried out only in highly equipped reference laboratories. (2) MLST in which the sequence of housekeeping genes mostly (sometimes the sequences of multiple virulence genes are used in MLST) will be compared and analyzed. (3) Multilocus variable Number Tandem Repeat Analysis (MLVA) which compares the number or size of certain DNA amplicons/repeats of polymorphic genes, intergenic spacers, or other DNA fragments [12]. (4) Amplified Fragment Length Polymorphism (AFLP) based on digestion of small amounts of genomic DNA with two restriction enzymes followed by ligation of linkers (adapters) to the genomic restriction DNA fragments before being subjected to PCR amplification using adaptor specific primers. The differences in the patterns obtained from various isolates within the same subspecies are attributed to mutations in the restriction sites or the insertion or deletion of DNA sequences within the amplified fragments [47,62], (5) Ribotyping, in which bacterial DNA is initially cut into (>300–500) small DNA fragments using restriction enzymes, differs from the PFGE which uses restriction enzymes that digest the genomic DNA in very few large fragments. The digested segments are separated by agarose gel electrophoresis followed by Southern blotting uses DNA probes labelled to detect he genes encoding the ribosomal RNA (rRNA), or (6) other techniques are less frequently used including the Randomly Amplified Polymorphic DNA (RAPD) [48], Plasmid profiling or the Restriction Fragment Length Polymorphism (RFLP). The choice of suitable technique depends mainly on the aim of the fingerprinting process, the cost of the technique, availability of infrastructure, time available, degree of discrimination required, ease of use, and the species of the investigated bacteria [17,62].

11. Future technology for the detection/prevention of mastitis

A new promising technique was developed which enables the amplification of the target DNA sequences in a different manner from PCR. The new technology, known as recombinase polymerase amplification (RPA), depends mainly on the use of two other proteins, beside the polymerase, which are the recombinase proteins and the single-strand binding proteins. The reaction is carried out under an isothermal condition. The used cycler has a clearly smaller size than the PCR thermocyclers which enables its application as a portable device. Many variants of RPA are also available such as the reverse transcription recombinase polymerase amplification (RT RPA), Multiplex RPA and on-chip RPA [45,63]. The new generation sequencers are becoming more cheaper and faster so that the whole genome sequencing will be often used in routine diagnostics in the near future in many diagnostic laboratories. A novel diagnostic technique was developed to detect the presence of *S. agalactiae* in milk. The Technique, known as LAMP (Loop-mediated isothermal amplification) was shown to be a rapid and economic diagnostic tool [64].

Another highly interesting futural tool is the analysis of certain circulating endogenous non-coding RNA molecules called Micro RNA (miRNA). It can be applied for early detection of pathogens invasion to the udder. The tool concept depends on the presence of high concentrations of exosomes in milk. It was found that the expression profile of at least 14 bovine microRNAs differ according to the health status of the udder. Therefore, the expression analysis of these RNAs can predict early udder infections [65–67].

Finally, it is worthy to mention that the use of molecular techniques is not only limited to the identification of the causative agents but extends to predict the possibility of udder infection in the future. Recently, strong evidence refers to the role mutations in *boLA* and *cxcr2* genes of cattle leading to bovine resistance to variable diseases of reproductive system such as mastitis, retained placenta and cystic ovaries [33]. Molecular analysis of certain mutations/ genotypes in cattle genome enables the prediction of the degree of the susceptibility/resistance of the cows to mastitis. Certain genotypes e.g. the analysis of the locus number G519663A in bovine Calcium channel, voltage-dependent alpha-2/delta subunit 1 gene (CACNA2D1 gene, present on the chromosome 4) [68,69], the 3 SNPs variants of the bovine breast cancer 1 gene (BRCA1) referred as G22231T, T25025A and C28300A in BBDDFF/AACCEE genotypes, cows harboring A-G SNP at nucleotide number 4525, intron 1 of TLR4 gene, the SNP rs132741478: g.2994A > G of the C4A gene (chromosome 8) and the ATP1A1 (chromosome 3), the CC and CA genotypes of ATP1A1 genotype, the genotypes T-35A, T-12G and G-102C of the gene PGLYRP-1 (chromosome 18), the CD14 gene (Chromosome 7) and the MBL-1 gene (Chromosome 28) [70–72].

12. Conclusions

The rapid and continuous development of molecular tools to cover different diagnostic needs enabled the PCR – and PCR dependent assays to replace the culture as a gold standard in the diagnosis of mastitis in farm animals. In addition to their role in mastitis diagnosis, they also support the mastitis control programs in different directions.

Competing interests

There is no conflict of interest.

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