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MOLECULAR GENETIC METHODS FOR IDENTIFYING RAW MATERIALS IN MEAT PRODUCTS: DIVERSITY, OPPORTUNITIES AND PROSPECTS

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Abstract
In the current economic situation, after easing the Covid pandemic restrictions, almost all laboratories, which are focused on evaluation of the conformity of food products, have faced issues in supplying for their laboratories. In this regard, in the last years many laboratories have been forced to validate new approaches and introduce new methods for assessing conformity of the food products. Very often it is not possible to use only one method to resolve the issue of the food product ingredients, especially for the purpose of traceability of their names and the used raw materials, listed on the label. Survey of the raw food materials to determine whether they correspond to the type name is a simpler task, in contrast to survey of the multicomponent food product. Many researchers have to estimate the opportunities and feasibility of application of various methodologies in their workplaces. Therefore, this review is relevant for the researchers in this field, as it focuses on aspects and special features of similar methodologies. The prospect of molecular genetic methods for identification of the raw materials used for manufacturing of meat products is presented below. This review also represents characteristics of methods for identification of the sources of raw materials used for the manufacturing of the meat products, based on the recognition of species-specific sections within the nucleic acids structures. The variety of methods (hybridization methods, polymerase chain reaction, different types of isothermal amplifications, methods using CRISPR/Cas systems), the principles of their implementation, and achieved analytical characteristics are considered. The capacities and competitive potential of various methods are discussed, as well as approaches being developed to overcome the existing limitations.

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Introduction

In recent years the intensification of interregional and interstate trade flows and the development of tracking information about manufactured and distributed commodities and products necessitate the methodical re-equipment of the tracking and control system to ensure compliance of food products with their declared composition. Unfortunately, the unfair producers remain interested in violating the declared composition of food products, including meat products, and using cheaper raw materials [1,2]. The significance of this issue goes beyond the incorrect informing of the consumers and unjustified increases in their expenses. Eating counterfeit meat products can be dangerous for health and also violate religious food restrictions [3–6].

In this regard there is necessity to expand the opportunities of obtaining data on the composition of meat products at all stages of their production and trade chains. It is extremely important that, along with the use of chromatographic, microscopic, electrophoretic and other analytical methods implemented in specialized laboratories and successfully solving the issues of confirmatory and arbitration control [7], the availability of simple and fast testing methods focused on widespread general laboratory equipment that does not require and special conditions for its implementation and professional training of the tests performers.

The observed dynamics of development of analytical methods proves the growing potential of molecular genetic assay as the methods of mass testing [8]. When solving issues of species identification, receptor molecules — oligonucleotides — are selectively bound due to complementary interactions of the nucleic acids contained in the tested samples, which are peculiar for the given organism. After this binding the following stages lead to the formation of intermolecular complexes, which include an enzymatic,

Table 1. Key review publications of the recent years on molecular genetic identification of the raw materials in meat food products

Article	Thematic specialization	Link
Authentication of meat and meat products using molecular assays: A review	General principles of molecular genetic methods, examples of their application	[9]
Market drivers and discovering technologies in meat species identification	Place of molecular genetic methods among other methods, diversity, integration with technological processes	[7]
A systematic review of DNA-based methods in authentication of game and less common meat species	Comparative evaluation of the development of different approaches based on bibliometric data	[10]
Current analytical methods for porcine identification in meat and meat products	Evaluation of molecular genetic methods in solving the problem of pork detection	[11]
Species identification and animal authentication in meat products: a review	Variety of molecular genetic methods, their comparison with alternatives	[12]
Authentication issues in foods of animal origin and advanced molecular techniques for identification and vulnerability assessment	Features of various practical problems, new methods	[2]

fluorescent, colloidal or the other type of the tags. The signal recorded due to this tag allows drawing a conclusion on presence in the sample of a biomaterial of the corresponding origin. In some cases the test result also includes a quantitative assessment of the content of the peculiar type of raw material [1,2].

Several reviews have been published describing the variety of already implemented developments and giving an idea of the principles of implementation and key differences between the various options of analysis — refer to the Table 1, summarizing the list of references and features of the material given in various reviews. However, against the background of the well-known polymerase chain reaction (PCR), as the historically first of the amplification methods for the selective detection of nucleic acids, the variety of methods still remains pretty poorly characterized, and the comparative evaluation of their advantages and disadvantages is not sufficient.

The purpose of this review is a unified comparative evaluation of the main groups of methods, description of their differences in their applicability for solving various issues, factors that limit the expansion of these methods, and the most promising directions for their prospective development. The properties of the main considered approaches and the results of applying the new methodological solutions are illustrated in the article with examples from the publications of the recent years, including the works of the authors.

Objects and methods

The object of the study was the developments of domestic and foreign scientists on the issues of molecular genetic control of the composition of meat products, presented in the articles and patents. The area of research included modern developments of analytical methods, features of their application in the identification of the species used as raw materials for the meat products. The search was run within the databases ScienceDirect, PubMed, Google Scholar, eLibrary, catalogs of patents of the Russian Federation, USA and EU, and the other publicly open electronic sources. Combinations of the keywords like control of the composition / ingredients, identification, detection, molecular genetic analysis, amplification analysis, hybridization methods, polymerase chain reaction, isothermal amplification, and non-amplification analysis were used. Keywords were used in English and Russian versions. In addition, thematically similar articles were searched also with the help of citation chains. Non-peer-reviewed, uninformative and duplicate sources were excluded from the results of the search; the same was done to the sources included in the search samples that were not related or just indirectly related to the topic of the research.

General issues in the development of molecular genetic analytical methods

The demand for nucleic acids as detectable targets is determined by the combination of conservative and varying (including species-specific) sectors within their structure, as well as the possibility to implement high-affinity interactions with complementary oligonucleotides, thereby ensuring high specificity of analytical methods [13,14]. DNA is present in all animal tissues and features very high stability when exposed to high temperatures during the production of meat food products [11]. These factors determine the using of DNA as a detectable target in the identification of raw meat materials.

When selecting target genes and DNA fragments as markers for evaluation the composition of products and identifying falsification, the conservation of the gene, its copy number, and the possibility of rapid extraction are taken into account. The listed requirements are well met by mitochondrial DNA (mtDNA) (regions of the mtDNA D-loop, cytochrome b genes (CytB), genes of subunits I, II and III of cytochrome c oxidase (COI, COII and COIII), genes of subunits 6 and 8 of adenosine triphosphatase (ATPase6 and ATPase8), genes encoding 12S and 16S ribosomal RNAs [15]. It is worth noting that mtDNA has a number of advantages compared to genomic DNA — more numerous copies, better accessibility for its isolation and the presence of conserved genome elements [16]. However, to detect counterfeits in the meat products, the markers related to genomic DNA are also used, for example, the genes for replication protein A1 (RPA1) [17], melanocyte-stimulating hormone receptor (Mc1r) [18].

Further in the review, the most significant approaches to DNA identification for the food industry are presented, reviewed and compared.

Hybridization methods

Hybridization plays a key role in detecting any type of DNA-DNA or DNA-RNA interactions [19]. The concept of nucleic acid hybridization was proposed in the 1950s, and in 1987 hybridization was used for the first time to identify the cooked meat [20,21]. Note that hybridization interactions occur in the course of all DNA identification methods, being necessary for recognition of target DNA. The hybridization methods discussed in this section differ from PCR and isothermal amplifications in that they are not accompanied by an increase in the number of target DNA copies. Therefore, these methods either use multicopy genes, or can detect rather high threshold levels of contamination, or should use special instrumental methods to ensure high sensitivity.

The mandatory stage of hybridization analysis is denaturation of the double-stranded DNA (dsDNA) target; usually it is thermal denaturation that takes place within the temperature range from 70 to 95 °C. After denaturation, specific recognition of the target by a complementary single-stranded DNA (ssDNA) probe occurs, carried out either in a heterogeneous or homogeneous format [22]. The formation of a complementary complex leads to the generation of a signal — electrochemical, fluorescent, colorimetric one (including visually detectable signal), etc. The scheme of the typical hybridization analysis is presented below in Figure 1.

In many developments nanoparticles with attached ssDNA probes are used to detect hybridization [23]. As far as identification of meat products is concerned, such methods demonstrate detection limits that are quite acceptable for practice: 6 μg/ml (pork) [24], 0.23 μg/ml (pork) [25], 4 μg/ml (pork) [26], 28 μg/ml (chicken) [27], 6 μg/ml (pork) [28], 12.3 ng/ml (horse meat) [29].

The principle of operation of this approach is well illustrated by the biosensor proposed by Ali et al. [25]. ssDNA was immobilized on the surface of gold nanoparticles (GNPs) to recognize pork gene fragment *CytB*pig mtDNA; The 3′-end was modified with a sulfhydryl group for immobilization on GNPs, the 5′-end was modified with tetramethylrhodamine (fluorescent label). The analyzed sample was heated to ensure denaturation of dsDNA, then incubated with the GNP-ssDNA hybrid structure. In the absence of the target fragment, the fluorescent label was located at the surface of the GNP and it showed no fluorescence. In the presence of a target DNA, the ssDNA probe attached to the surface of the GNP formed dsDNA with a complementary ssDNA target, which ensured distancing of the label and provided for fluorescence. The proposed biosensor made it possible to detect up to 1% pork in raw and heat-treated meat products. Due to using of short DNA fragment (27 bp) as a target, the analysis is possible even for samples that contain highly damaged DNA.

Hybridization methods serve as the basis of DNA microarrays, which are the clusters of dots on silicon or glass substrates with an ordered arrangement of ssDNA probes that differ in nucleotide sequence for target DNA recognition [30]. The detection limits of hybridization DNA microarrays range from 0.1% to 0.01% [31].

For the hybridization approach, the influence of processing of meat products on the identification of raw materials (chicken, pork, beef and horse meat) was evaluated [25,32]. It was shown that thawing and freezing did not lead to a significant decrease in hybridization. When exposed to high temperatures — 100–120 °C — signals fading was observed due to degradation of DNA, but the raw material remained identifiable. The type of analyzed tissue and pretreatment at high temperatures provided the great-

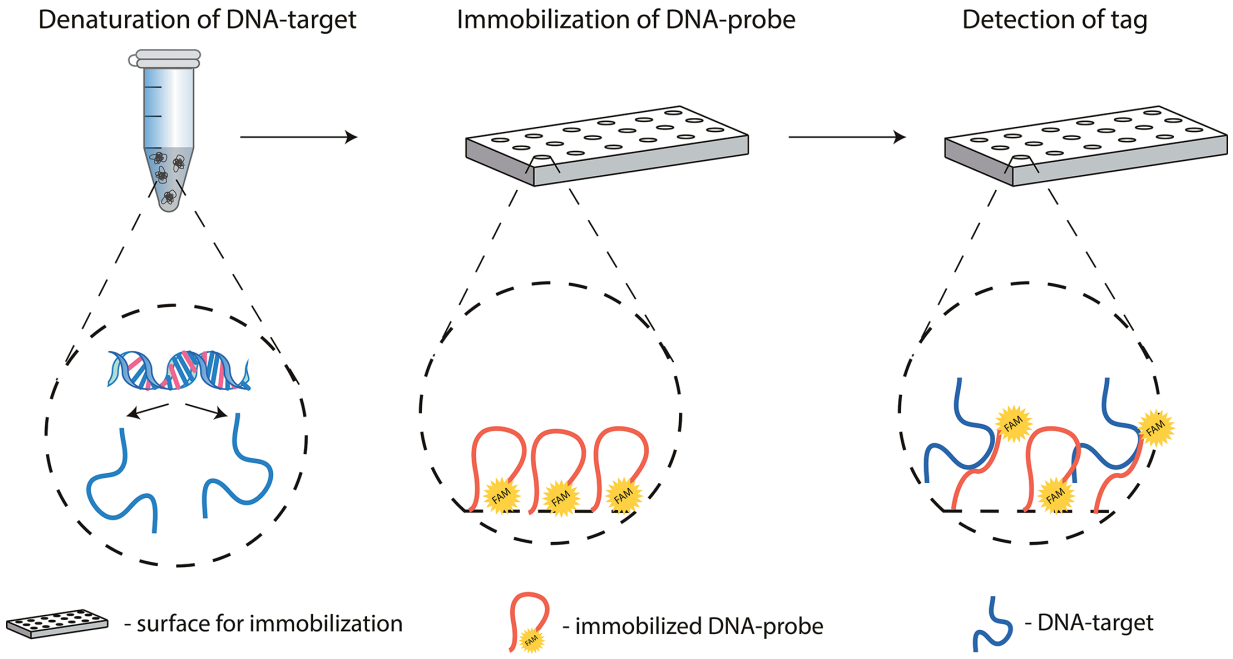

Figure 1. Hybridization analysis scheme (Source: Compiled by the authors)

est influence on the efficiency of hybridization analysis. Changing storage conditions had a limited effect, with the exception of storing meat at room temperature. The authors concluded that DNA hybridization provides a reliable basis for detecting animal species used in most meat products when the meat share exceeds 5%.

Summarizing the discussed results, we can conclude that DNA hybridization is effective for identifying the meat products. The use of nanomaterials opens up the new opportunities for more convenient, effective and low-cost application of this approach.

Methods based on polymerase chain reaction (PCR)

Today PCR and its variants are the most often used methods to detect meat and meat products counterfeits. It is explained by the sensitivity, simplicity and reliability of this method. PCR is based on increasing the number of copies of the target DNA through repeated cycles: high-temperature denaturation of the target DNA (94–98 °C), annealing of the primers on complementary single-stranded fragments of the target DNA (DNA-DNA hybridization) (50–64 °C), elongation — the polymerase synthesis of the DNA chain following the primer (72–80 °C). This canonical pipeline makes possible to produce multiple copies of the target DNA (amplicons) due to precise regulation of temperature. To visualize amplicons, it is possible to use electrophoresis in agarose gel (an approach that requires additional time and labor), fluorescent staining, or immunochromatographic tests (ICT). The latter option is a simple and promising approach that requires the use of a pair of primers with tags that shall be recognized by ICT with high specificity and sensitivity [33]. Figure 2 shows a scheme of the ICT for amplicon detection. Thus, visualization of PCR products using ICT made it possible to increase sensitivity by 10 times in comparison with gel electrophoresis and significantly reduce duration of the analysis [34–36].

Among DNA-based meat identification methods, the most popular and widespread are the following ones:

- PCR with endpoint analysis of the results. The results are recorded at the end of the PCR. Intercalating dyes or hybridization probes with a fluorescent label can be used as fluorophores [38]. However, the significant drawback is the inability to estimate the increment of

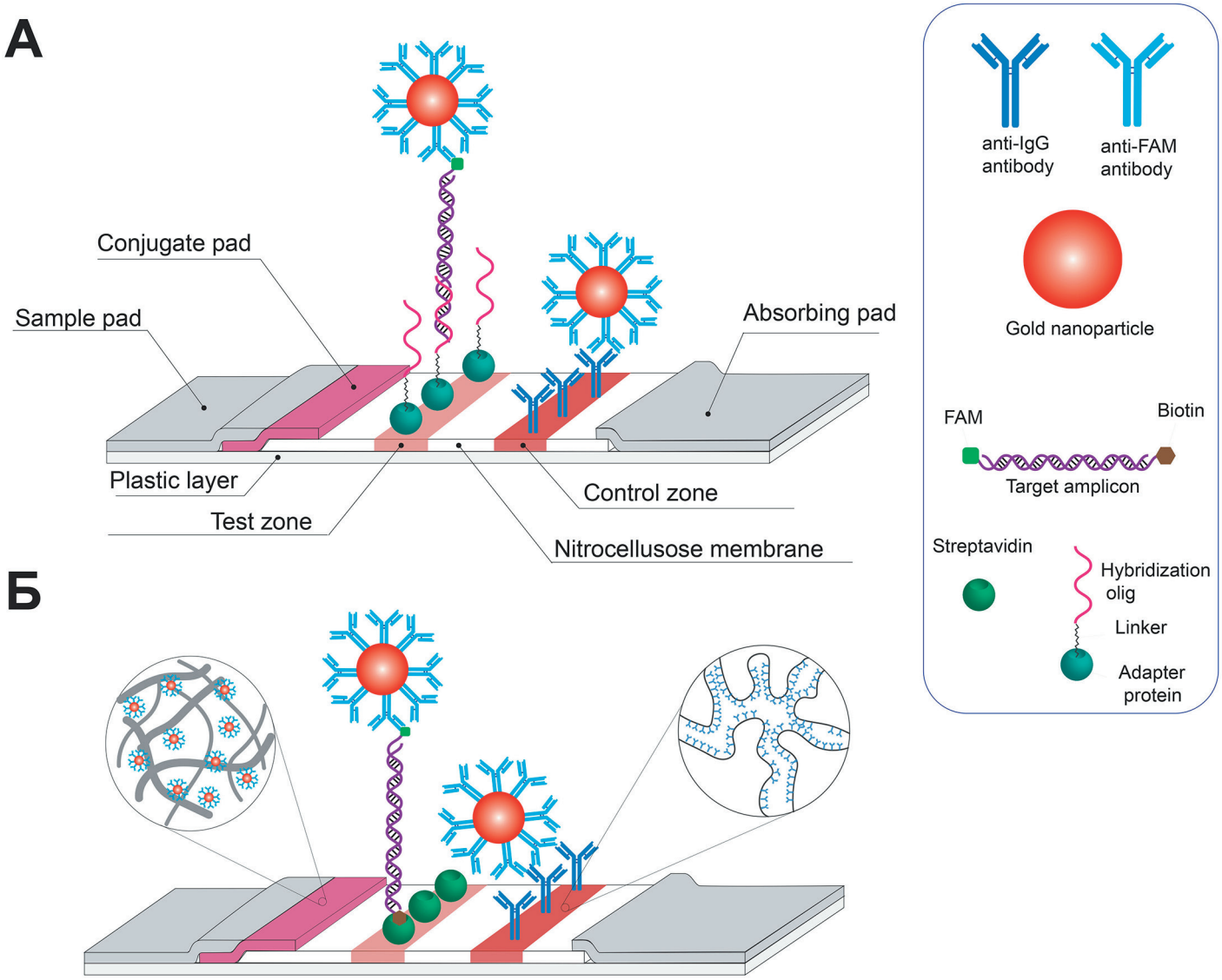

Figure 2. Scheme of an immunochromatographic test for the detection of amplicons (based on [37], with changes). FAM — fluorescein label

the fluorescent signal per unit of time at different stages of amplification, which can cause false positive results;

- Real-time PCR using SYBR Green. The fluorescent signal is detected during the amplification process [39–43]. SYBR Green is the most common intercalating dye. The drawbacks are the ability of SYBR Green to get bound to any dsDNA, and its inhibitory effect on the polymerase;
- Real-time PCR with TaqMan probe. The fluorescent signal is detected during the amplification process. In addition to the primers, the reaction mixture contains a hybridization DNA probe (TaqMan), complementary to the target DNA with fluorophore and fluorescence quencher at the opposite ends. DNA polymerase features exonuclease activity, due to which the annealed TaqMan probe is cleaved during the elongation stage, and fluorescence is recorded [44];
- multiplex PCR is the simultaneous amplification of two or more DNA targets in one tube. It is implemented by several pairs of primers, each of which is specific per one target [41,45–47]. Its efficiency depends on the specificity, copy number of each DNA target, and selection of the annealing temperature that is optimal for all primers' pairs;
- digital PCR in droplets. The PCR reaction mixture is sprayed into tens of thousands of tiny droplets. One microdroplet hits either one target or none. PCR takes place in the droplets (Figure 3 illustrates the principle of the analysis) [48–50]. The method allows determining the absolute concentrations of nucleic acids without the application of calibration curves. To implement the method, an emulsion generator and an amplifier are required.

These and other types of PCR are used to detect counterfeits in meat and meat products, thus showing high specificity, sensitivity and speed.

Isothermal amplification methods

For the recent years several alternatives to PCR have emerged that also increase the number of copies of the original target DNA, but capable to run at the same temperature. All methods that fit this definition are called isothermal amplification methods [37,51,52]. Isothermal methods do not only make amplification easier, but also allow detecting the product using simple tools and instruments, for example, membrane test strips — ICT (the scheme of a typical analysis that combines isothermal amplification and ICT is shown in the Figure 4).

More than ten types of isothermal amplification are known, the main ones are listed in the Table 2. The most common and promising types are recombinase polymerase amplification (RPA) [53], loop-mediated isothermal amplification (LAMP) [54], and rolling circle amplification (RCA) [55]. These methods can be implemented at a single temperature (LAMP ~60 °C, RPA and RCA ~37 °C) within 10–60 minutes depending on the sequence being recognized and the type of amplification. LAMP, RPA and RCA have been described with the very high score of sensitivities that are not worse than PCR and allow detecting single copies of nucleic acids. At the same time, each of these methods has advantages and disadvantages.

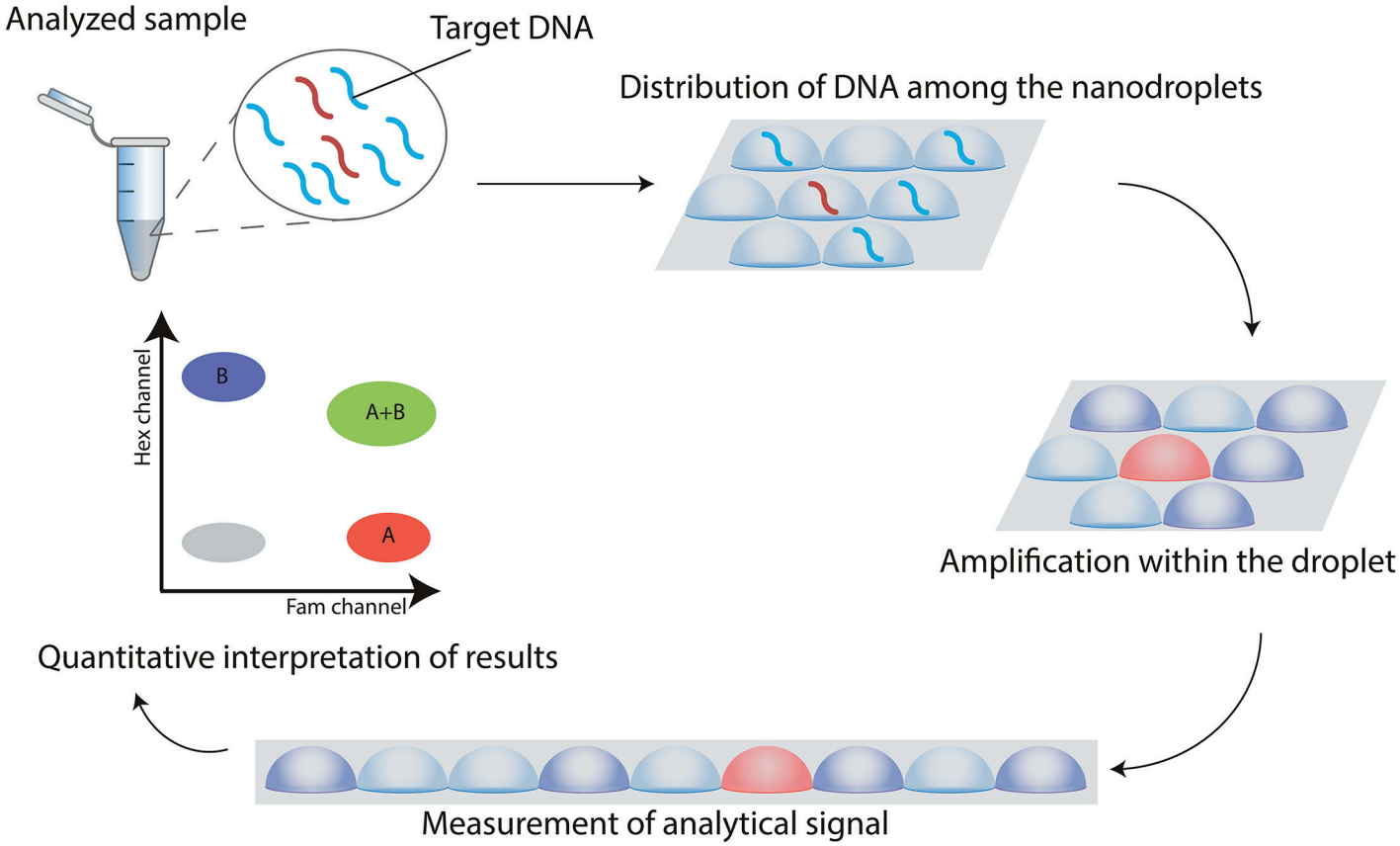

Figure 3. The principle of digital PCR in the droplets (Source: Compiled by the authors)

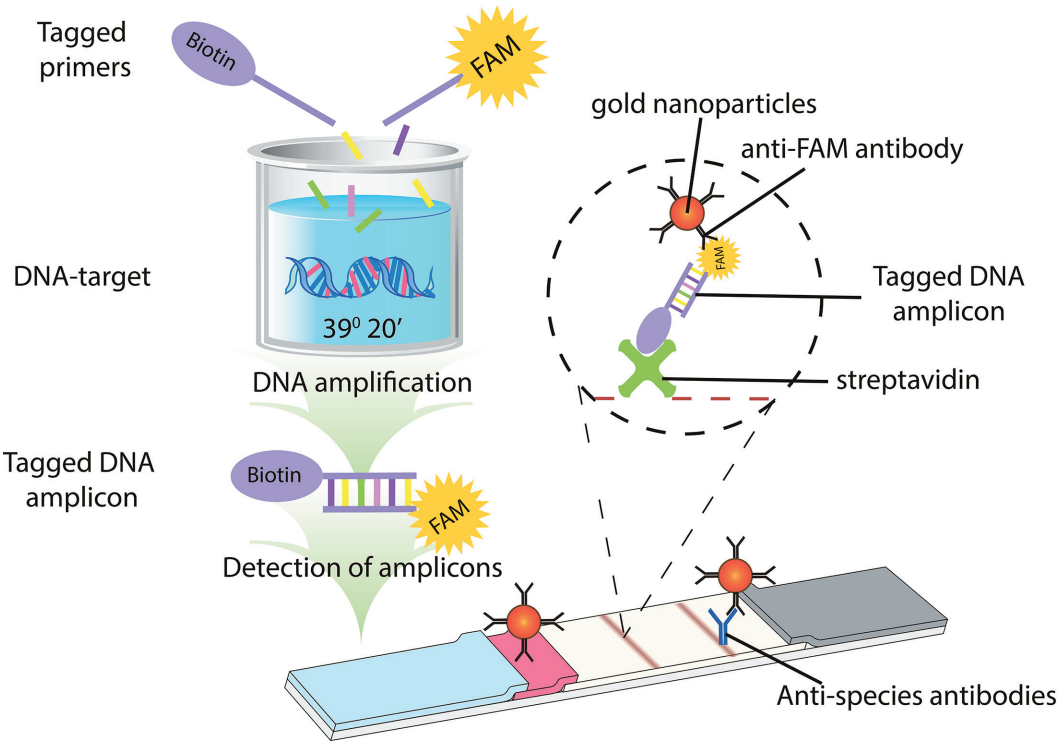

Figure 4. Scheme of an assay combining isothermal amplification and ICT fluorescent label (Source: Compiled by the authors)

Table 2. Comparison of isothermal amplification methods performances (based on [37])

Amplification type*	LAMP	RPA	RCA	NASBA	HDA	CRISPR/Cas
Parameter/property						
Temperature, °C	60	37	30	42	37	37
Duration, min	30–60	10–30	200–240	60–120	60–120	20–30
Specificity	+++	+	++	++	+++	+++
Sensitivity	+++	++	++	++	++	+
Non-laboratory analysis	+++	+++	+	+	+++	+++
Multiplex analysis	+	+++	++	++	+++	++
Resistance to inhibition	+++	+++	+	+	+++	++

* LAMP — loop-mediated isothermal amplification, RPA — recombinase polymerase amplification, RCA — rolling circle amplification, NASBA — nucleic acid sequence-based amplification, HDA — helicase-dependent amplification.

Thus, RPA is the simplest to implement, requiring only one pair of primers and a commercially available reaction mixture (manufacturer TwistDX, UK). As a result of RPA, homogeneous dsDNA product of a given length is obtained. The successful application of RPA in combination with fluorescent and colorimetric detection [56,57] or with ICT [58–65] for detecting the impurities in meat (chicken, duck, pig, etc.) has been described.

RCA reproduces circular DNA as multiple linear copies. The process starts with the presence of one primer and Phi29 DNA polymerase. RCA has great potential due to the variety of options for obtaining circular DNA by ligation [66]. This approach, combined with SYBR Green I fluorescent staining, was used to detect horse meat in beef food products (cytB gene) (limit of detection (LOD) = 63 ng/ml, 0.01% of horse meat in beef food) [67].

LAMP requires two or three pairs of primers and leads to the formation of fragments of various lengths [68,69]. LAMP is isothermal amplification, which is most widely used for identification of meat products. LAMP products can be determined by various methods, including recording the turbidity in the reaction mixture [70], running gel electrophoresis [71], measuring the fluorescence of a dye incorporated into DNA (intercalating) [72], indicating via metals ions [73,74], and monitoring changes in pH [75], detection based on the formation of pyrophosphate, visual detection using test strips [76], etc. Detection of LAMP products based on gel electrophoresis, turbidity measurements and intercalator fluorescence, despite examples of successful use for detecting the counterfeited food products [77], have limited prospects due to its duration or subjectivity in evaluation of the results. The use of primers with fluorescent labels significantly speeds up the detection of LAMP amplification products. So, Qin et al. [78], using LAMP in combination with fluorescence polarization to detect undeclared admixture of pork in beef food products showed high specificity and sensitivity of the 30-minute analysis.

pH-sensitive indicators such as phenol red, cresol red, neutral red and m-cresol violet can also be used to monitor the formation of DNA amplicons in LAMP. LAMP processes are accompanied by the accumulation of H^+ and, accordingly, decrease of pH in the reaction solution [79]. Thangsunan et al. [75] used neutral red as a pH indicator in LAMP with colorimetric detection in order to detect counterfeit in raw and processed meat products, which allowed achieving limit of detection (LOD) of 0.01% in poultry meat.

DNA targets amplified by LAMP can be visually detected with the help of fluorescent dyes or colorimetric tests. However, operator's interpretation of may lead to errors due to the turbidity of the tested sample or ambiguity in color changes. ICTs that detect amplification products with tags introduced through primers (see Figure 2 and Figure 4) minimize the subjectivity of evaluation of the results due to clear visualization of the test zone and control zone [80]. For example, Jawla et al. [81] developed a method combining ICT and LAMP for detecting the counterfeited admixtures in beef food products. Registration of its results via ICT provided the high sensitivity of this method [81].

Isothermal amplification methods deny the need for an amplifier, which allows for simple and fast out-of-laboratory analysis [51,72,77]. The preference of LAMP for identifying the authenticity of meat products in comparison with the other isothermal methods is explained by its low cost and the availability of enzymes. The main disadvantage of LAMP is the interactions between the 4–6 primers used, which can cause the formation of a nonspecific DNA product.

CRISPR/Cas system methods

Another promising tool for specific identification of meat products is Cas endonuclease-based recognition, the clustered regularly interspaced short palindromic repeats (CRISPR). CRISPR/Cas is a bacterial adaptive immune system but it can also be used for *in vitro* diagnostics. The discovery of collateral DNase activity (trans-cleavage) for Cas12a endonucleases has become the pivot point for diagnostics [82]. The principle of this approach is based on the acquired nuclease activity of Cas12a — when the Cas endonuclease is included into the complex with guide RNA (CRISPR RNA, gRNA), then if there is a dsDNA target (cis-target) complementary to the gRNA region in the sample, its recognition and cleavage occurs (cis-cleavage). Moreover, Cas this way acquires the ability to perform off-target collateral cleavage of any ssDNA (trans-targets). Over the past years, Cas endonucleases of various families (Cas9, Cas12, Cas13 and Cas14), that differ both in the structure of the protein and the structure of the required gRNA, and in the types of recognized cis-targets and trans-targets, have proven their efficiency for identifying DNA/RNA targets with high selectivity, being capable to recognize even the sequences with single nucleotide substitutions [83].

The CRISPR/Cas approach has also proven its efficiency in detecting adulterated meat products. Wu et al. used the CRISPR-Cas12a system to rapidly (30 min) detect pork DNA in mixture samples at 37 °C without prior amplification [84]. However, CRISPR/Cas systems, being used on its own only, often fail to achieve the required sensitivity. Therefore, to detect nucleic acids, they are supplemented with the other approaches, in most cases preliminary isothermal amplification is performed (Figure 5). Liu et al. combined Cas12a with RPA to detect adulterated beef, pork

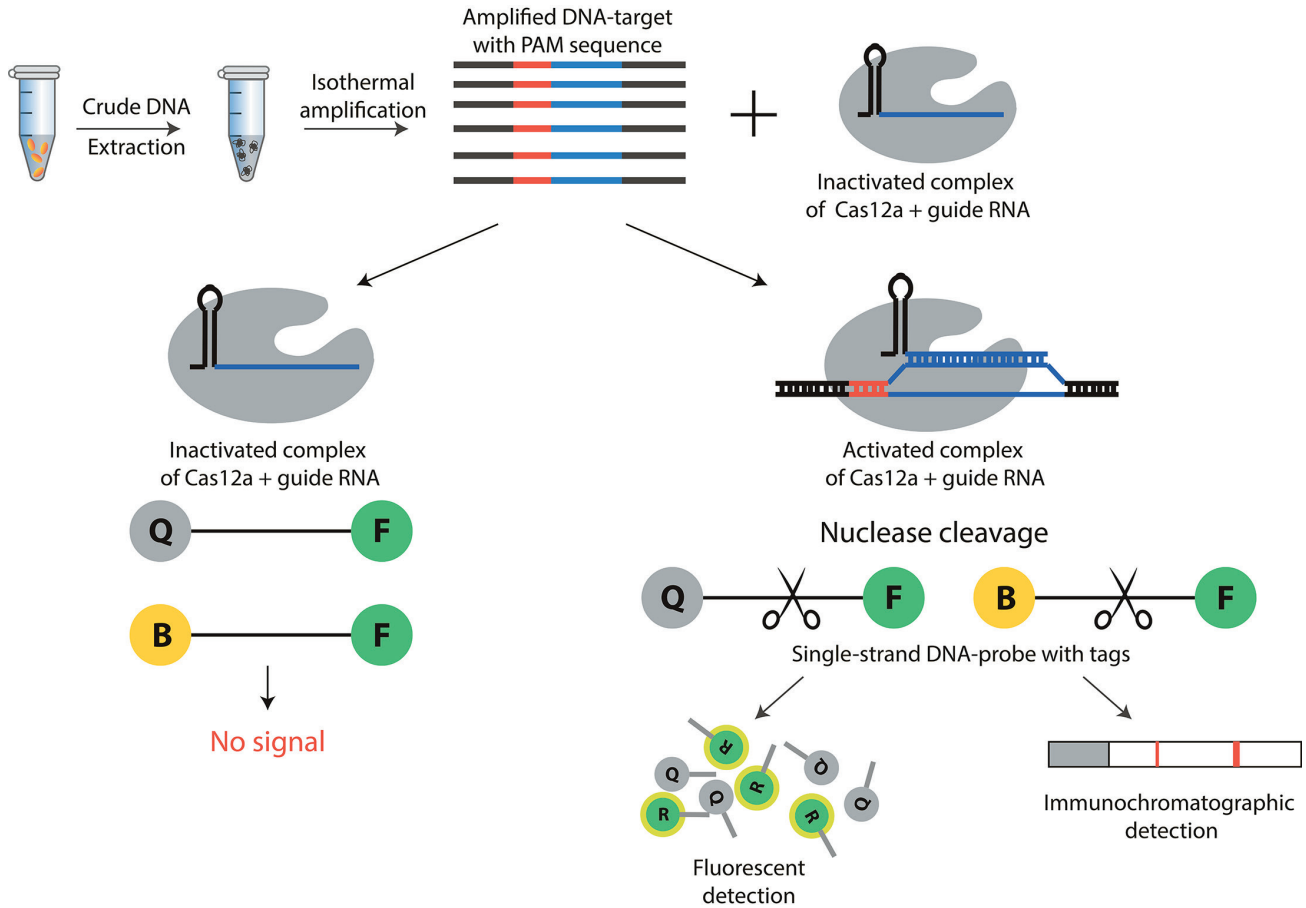

Figure 5. Scheme of analysis, including isothermal amplification, recognition of amplicons by the system CRISPR-Cas12a, fluorescent or immunochromatographic detection of single-stranded DNA probes cleaved by activated Cas12a
(Source: Compiled by the authors)

and duck meat (RPA-Cas12a-FS), achieving the limit of detection per 10 copies in 45 minutes [85]. In the research of Zhao et al. [86] the combination of RPA and CRISPR/Cas12a allowed detecting pork in the food composition, with a detection limit of up to 10^{-3} ng within 30 minutes, and also detect up to 0.1–0.001% share of pork in the meat products that were subjected to freezing, boiling and autoclaving.

In comparison with the other technologies the application of CRISPR/Cas systems requires minimal laboratory equipment, which significantly increases the efficiency of nucleic acid detection and the practical applicability of this approach. Disadvantages of CRISPR/Cas systems include the necessity for preliminary amplification to achieve high sensitivity. This necessity increases the risk of contamination and increases the duration of the analysis. Further progress, of this approach is associated with the development of a single-stage "closed" system that provides the necessary sensitivity. Nowadays only Cas12a has been used to detect adulterated meat products, while the capabilities of other Cas family proteins still remain undefined.

Molecular genetic methods are actively used to test and control the composition of meat food products. The fundamental feature of these methods is the absence of necessity for complicated instrumentation, as the testing does not involve fractionation and identification of sample components, but is limited to the registration of a tagged specific complex. To date, a number of commercialized analytical kits and methods is available and is included into the official recommendations of state and international regulatory authorities. However, the capabilities of molecular genetic testing are not fully implemented in practice. Actively progressing new developments, primarily related to isothermal amplification, move towards the autonomous analytical systems that can be used beyond the specialized laboratories. That significantly reduces labor intensity of testing and strives to autonomously functioning systems [87,88].

Conclusion

The aspects of molecular genetic research methods considered in the review are not the new direction in the field of test and control in the global laboratory practice. But they are practically not used in the Russian Federation, of course, with the exception of the PCR-based method. In connection with this, in our country all the considered methodologies have not yet been standardized. As a result of review of the recent publications, the characteristics and performance of the major considered approaches and the results of applying new methodological solutions were systematized. The analysis presented above will undoubtedly be useful for orientation in the current state of development of molecular genetic methods aimed at the species-specific identification of components in the meat products.

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