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Comparative analysis of alternative and standardized microbiological diagnostic methods used in food expertise

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Abstract

For the comparative analysis of the results of the alternative and the standardized reference methods used in microbiological food testing, we tested 7761 samples for the detection of foodborne pathogens (Salmonella spp., Listeria spp., Campylobacter spp) and 5123 samples for the detection of quality indicators (Coagulase Positive Staphylococcus, Escherichia coli, Enterobacteriaceae, Total Aerobic Germs, Yeasts and Molds), using Mini Vidas equipment and TEMPO, respectively. Non-compliant samples resulting from food screening by alternative methods were subsequently tested by standardized reference methods to establish the correlation of the results. It has been found that fast alternative diagnostic methods used to detect food pathogens generate results equivalent to those of standardized reference methods, and rapid diagnostic alternative methods for detecting quality indicators generate results with a correlation of 98.38%. The bacterial identification achieved by comparative use of the alternative and the reference methods, namely Vitek 2 Compact and Kaufmann - White, may show differences in the obtained results, the correlation being for the Salmonella genus of 77.8%, and, in the case of Listeria genus, 100%.

Keywords: *alternative method, standardized method, pathogenic germs*

1. Introduction

Conventional bacterial testing methods rely on specific media to enumerate and isolate viable bacterial cells in food. These methods are very sensitive, inexpensive and can give both qualitative and quantitative information on the number and the nature of microorganisms present in the food sample. Traditional methods for the detection of bacteria involve the following basic steps: pre-enrichment, selective enrichment, selective plating, biochemical screening and serological confirmation. (PUCHIANU GHEORGHE, BABII MIHAELA, NECULA VALENTIN AND ENACHE DORIN VALTER [9])

Hence, a complete series of tests is often required before any identification can be confirmed. These conventional methods require several days to give results because they rely on the ability of the organisms to multiply to visible colonies. Moreover, culture medium preparation, inoculation of plates and colony counting makes these methods labor intensive. Conventional methods, generally regarded as the golden standard, often take days to

complete the identification of viable pathogens. Any modification that reduces the analysis time can technically be called rapid method. (P.K. MANDAL, A.K. BISWAS, K. CHOI, AND U.K. PAL [5])

Rapid and novel methods in microbiological tests provide more sensitive, precise and reproducible results compared with conventional methods. NEMATI M, HAMIDI A, MALEKI DIZAJ S, JAVAHERZADEH V, LOTFIPOUR F. [6], VAN DER ZEE H. [8]).

A rapid method can be an assay that gives instant or real time results, but, on the other hand, it can also be a simple modification of a procedure that reduces the assay time. These rapid methods not only deal with the early detection and enumeration of microorganisms, but also with the characterization of isolates by use of microbiological, chemical, biochemical, biophysical, molecular biological, immunological and serological methods. P.K. MANDAL, A.K. BISWAS, K. CHOI, AND U.K. PAL [5])

The food industry is in need of more rapid methods which are sensitive for the following reasons: to provide immediate information on the possible presence of pathogen in raw material and finished products; low numbers of pathogenic bacteria are often present in complex biological environments along with many other non-pathogenic organisms; the presence of even a single pathogenic organism in the food may be a infectious dose; for monitoring of process control, cleaning and hygienic practices during manufacture; to reduce human errors and to save time and labor cost. (P.K. MANDAL, A.K. BISWAS, K. CHOI, AND U.K. PAL [5])

The reviewed literature showed that rapid methods and automation in microbiology is an advanced area for studying and applying of improved methods in the early detection, and characterization of microorganisms and their products in food, pharmaceutical and cosmetic industries as well as environmental monitoring and clinical applications (NEMATI M, HAMIDI A, MALEKI DIZAJ S, JAVAHERZADEH V, LOTFIPOUR F. [6])

Such methods are now available for rapid detection or estimation of groups of (indicator) organisms, pathogenic micro-organisms, bacterial toxins and mycotoxins, and molds. These alternative methods can be classified by the principles on which they are based: modified conventional methods, instrumental measurement of bacterial metabolism, bioluminescence, immunological techniques, DNA techniques and combinations of these techniques. To meet user expectations, test kits must be accurate, sensitive, specific, rapid (24 h or less), easy to use, and labor-saving. They must also offer the possibility of computerization, a low detection limit, and low investment and running costs (VAN DER ZEE H, HUIS IN'T VELD JH [7], VAN DER ZEE H, HUIS IN'T VELD JH [8], GUILLERMO LÓPEZ-CAMPOS,

There is a continuous development of methods for the rapid and the reliable detection of food borne pathogens. Advent of biotechnology has greatly altered food testing methods. Improvements in the field of immunology, molecular biology, automation and computer technology continue to have a positive effect on the development of faster, more sensitive and more convenient methods in food microbiology. (P.K. MANDAL, A.K. BISWAS, K. CHOI, AND U.K. PAL [5], JOAQUÍN V. MARTÍNEZ-SUÁREZ, MÓNICA AGUADO - URDA, VICTORIA LÓPEZ-ALONSO [3])

To meet future challenges in an always changing environment, appropriate expertise needs to be identified and a model of specialized and continuous training is required. (SHAPING THE FUTURE OF FOOD SAFETY, TOGETHER [11]).

After 48h of incubation, the PCR method in all cases showed equal or better results than the reference cultural Food and Drug Administration, for example in the case of detection of *L. monocytogenes*. (GEORGE P. PATRINOS, WILHELM ANSORGE, PHILLIP B. DANIELSON [2]).The independent validation study examined by Campden and Chorleywood Food Research Association Group (CCFRA) demonstrated that the LightCycler *L. monocytogenes* detection system shows a comparable sensitivity to reference methods.

(JUNGE B, BERGHOF-JÄGER K. Roche/BIOTECON [4], RONALD G. LABBÉ, SANTOS GARCÍA [10]).

A major disadvantage of alternative and rapid methods over cultural methods is that most methods need damaging of the cells and therefore, viable cells for result confirmation and further characterization can only be obtained by repeating the analysis using standard cultural procedures. Moreover, rapid methods usually detect only one specific pathogen, while cultural methods simultaneously detect and isolate many pathogens by including several types from numerous microbiological examinations or samples, selective media in the analysis. The use of several rapid assays to do multi pathogen analyses on a food sample makes this analysis unacceptably expensive. (P.K. MANDAL, A.K. BISWAS, K. CHOI, AND U.K. PAL[5], BLAKE W. BUCHAN, NATHAN A. LEDEBOER [1])

It is also to be noted that in accordance with the Commission Regulation (EC) 2073/2005 of 15 November 2005 regarding microbiological criteria for food products, food business operators must be able to use other analytical methods than those reference methods, in particular faster methods as long as alternative methods give equivalent results. (COMMISSION REGULATION (EC) 2073/2005 [12]).

2. Materials and Methods

The purpose of the this paper was to verify the correspondence of the results of rapid alternative diagnostic methods with those of the standardized methods, by examining food samples taken during self-control programs from different processing units within Braşov County.

The samples were originally tested by alternative methods and those found with positive (non-conforming) results were then analyzed by standardized methods in order to confirm/refute the obtained results.

The exams performed by alternative methods were:

- the detection of food pathogens (*Salmonella spp*., *Listeria spp*., *Campylobacter spp*) by using the Mini Vidas equipment;
- the enumeration of the quality indicators (*Coagulase Positive Staphylococcus*, *Escherichia coli*, Enterobacteriaceae, Total Number of Aerobic Germs, Yeasts and Molds) using the TEMPO equipment;
- the bacterial identification using the VITEK 2 COMPACT equipment.

The reference methods used to confirm the positivity cases resulted from the use of fast alternative diagnostic techniques were the standardized ones, as follows:

- the detection of food pathogens: SR EN ISO 6579 - 1 (*Salmonella spp*.); SR EN ISO 11290-1 (*Listeria spp.*); SR EN ISO 10272-1 (*Campylobacter spp*.),
- the enumeration of the quality indicators: SR EN ISO 6888-2:2002/A1:2005 (*Staphylococcus spp*.); SR ISO 16649-2:2007 (*Escherichia coli*); SR EN ISO 21528-1,2 (Enterobacteriacee); SR EN ISO 4833-1/2014 (Total Number of Germs); SR EN ISO 21527-1 (D + M);
- the Kaufmann – White method, for the bacterial identification.

The expertise was carried out within the Sanitary Veterinary and Food Safety Laboratory of Brasov, using validated and accredited methods in accordance with SR EN ISO 17025/2005, both for the reference methods and the alternative methods. The bacterial identification by the Kaufmann-White method was performed at the Institute of Hygiene and Veterinary Public Health Bucharest.

For the detection of food pathogens, we tested a number of 7761 samples using the Mini Vidas, as follows: (Table 1)

Table 1: Number of samples taken and examined by alternative techniques for detection of food pathogens

Analized parameters	Samples	
	No.	%
Salmonella spp.	4257	54,85
Listeria spp.	2983	38,44
Campylobacter spp.	521	6,71
TOTAL	**7761**	**100,00**

The Mini Vidas equipment is a compact high-performance system in the identification of highly pathogenic germs (*Salmonella spp.*, *Listeria monocytogenes*, *Campylobacter jejuni*, *Staphylococcus enterotoxin*, etc.), having the immunological analysis as working principle.

Compared to the standardized methods, its use allows time savings in the preparation of culture media, glass sterilization, packaging, labeling, inoculation, plate readings, autoclaving and glassware washing, using ready-to-use reagents. (Fig. 1)

Figure 1. MiniVidas Automated Analyzer

The MiniVidas Automated Analyzer offers a wide range of analyzes, applications, safety and ease of use, being a automated, standardized, robust device that allows objective reading and delivering a quick result compared to classic workflows.

Matrices from which food pathogens can be identified and the time required for laboratory diagnosis for the detection of foodborne pathogens are: (Table 2)

Table 2. Matrices and required times

Bacterial germs that can be identified	Matrice	Required times (hours)
Salmonella spp.	All food products during their validity	48
Lsteria monocytogenes	All food products before leaving the direct control of the unit which produced them and products marketed during their shelf life (meat and meat products, raw milk, unpasteurized milk cheeses, confectionery and pastry products, food, fish and fish products)	72
Campylobacter jejuni	Chicken carcasses - surveillance period 1.04-30.09. (warm season).	72

To quote the quality indicators, we have tested 5123 samples using the TEMPO equipment, as follows: (Table 3)

Table 3. Number of probes sampled and examined by alternative techniques for the

enumeration of quality indicators

Analized parameter	Number of samples	
	No.	**%**
Staphylococ c.p.	855	16,69
E. coli	1235	24,10
Enterobacteriaceae	943	18,41
NTG	1520	29,67
D + M	570	11,13
TOTAL	**5123**	**100,00**

The TEMPO method is a fully automated method that allows the quantitative determination of bacterial germs. It is based on traditional microbiology, based on the multiple tube method. Unlike standardized methods, it has a number of advantages, of which the most important are: sensitivity and ease of use, delivering a quick result, saving time in preparing media, preparing for glass sterilization, packaging, labeling, inoculation, plate readings, autoclaving and glassware washing, etc. (Fig. 2)

Figure 2. Reading station: Reading, interpreting, validation and transfer of results.

The TEMPO test consists of a card with a transfer tube and an ampule with a specific culture medium. Culture media are desiccated, sterile, ready-to-use, disposable, selective: TC (Total Coliforms); EC (*E. coli*); EB (Enterobacteriaceae); STA (*Coagulase Positive Staphylococcus*); LAB (Lactic Acid Bacteria) or non-selective: TVC (NTG); Y + M (Yeasts and Molds), identified by bar code and color code, and Stomacher TEMPO sachets.

The medium is inoculated with a dilution of the sample to be tested and transferred by the tempo filling instrument into the tempo card. The medium is homogeneously dispersed in 48 wells with three different volumes. The card is then hermetically sealed to avoid any risk of contamination during manipulations. Later, the interpretation, the validation and the transfer of the results take place in a single stage. (Fig. 2)

Fig. 4. Card with a transfer tube and an ampoule with a specific culture medium .

Example:

Tempo TVC and Tempo EC - during incubation, the microorganisms present in the card reduce the substrate from the culture medium and cause a fluorescent signal to be detected by the TEMPO reader. Depending on the number and size of the positive wells, the tempo system deducts Total Number of Germs (for the Tempo TVC test) or the *E.coli* number (for the EC test) present in the initial sample, based on the most probable number method.

Matrices from which bacterial quality indicators can be identified and the time required for laboratory diagnosis for the detection of foodborne pathogens are: (Table 4)

Tabelul 4. Matrices from which bacterial quality indicators can be identified and the time required for laboratory diagnosis for the detection of foodborne pathogens

Bacterial germs that can be identified	**Matrice**	**Required times (hours)**
E.coli	Meat products, mechanically separated meat, heat-treated milk cheeses, non-dairy products.	24
Staph. c.p.	Raw milk cheeses subjected to lower heat treatment than pasteurization and heat-treated milk, milk powder, fish products.	24
Enterobacteriaceae	Cattle, sheep, goats, horses, pigs, pasteurized milk and pasteurized dairy products, milk powder, ice cream and dairy desserts, infant formula and food for medical purposes, egg products.	48
NTG	Milk raw material, carcases of cattle, sheep, goats, pigs, horses, poultry, minced meat and mechanically separated.	48
D + M	Bakery products	72

Some of the non-conforming samples found during the detection of food pathogens (12 strains of *Salmonella spp*. and 9 strains of *Listeria spp*.) were reexamined with the Vitek 2 Compact and Kaufmann-White methods in order to confirm the initial results.

Vitek 2 Compact is an automatic biochemical and antibiotic confirmation system capable of selecting pathogenic and highly pathogenic organism isolates on solid media by performing biochemical tests in a very fast time, resulting in time savings in generating the results. (Fig. 5)

Figure 5. Vitek 2 Compact

By using it, we save materials and reagents. Thus, the used card saves all types of reagents required for the identifications and the confirmations and it does not require any type of glassware, thermal sterilization, thermostatic or other pre-diagnostic steps.

It's an easy-to-use device with a low work-time, intuitive software and connection to the computer system. The method is fully automated, it uses different diagnosis cards to identify Gramm + (GP), Gramm - (GN), Anaerobic bacteria (ANC), Campylobacteria (NH), Corynebacteria (CBC), Yeast and Mold (YST) and *Bacillus* (BCL).

The working stages are the following: calibration of the apparatus by using specific kits for each methodology, inoculation of the pathological material from the previously purified microbial cultures into kit strips, determination of the bacterial optical density (OD) by means of a device called DENSIMAT (densitometer) specific to each bacteria genus, attachment of the tube with the bacterial density determined on the card specific to the method, inserting the side tube of the card into the glass tube with established bacterial optical density, inserting the card with the glass tube attached to the mobile carrier of the apparatus, closing the hatch, processing the sample identification data, assigning a sample code, requesting the genus to which the micro-organisms to be tested belong, validating requests and starting the apparatus. (Fig. 6)

Example: *Salmonella spp.*

Fig. 6. The main steps for identifying bacterial germs using the Vitek 2 Compact

After a variable period of time, the equipment generates a printed report where the biochemical properties of the identified bacteria genus and the probability of its validation are

indicated. Examples: *Salmonella spp*., 5-6 hours; *Listeria monocytogenes*, 6-8 hours, *Campylobacter spp*., 8-12 hours; *Escherichia coli*, 4-6 hours; *Staphylocuccus spp*., 4-6 hours, Yeast and Mold, 4–6 hours.

3. Results and discussion

After analyzing the 7761 samples for the detection of food pathogens and the 5123 samples for the detection of quality indicators by fast alternative diagnostic methods, the following results were obtained: (Table 5 și 6)

Table 5. Results obtained from the detection of food pathogens through fast alternative diagnostic methods

Analized parameters	**Samples No.**	**Results**			
		Noncompliant		**Compliant**	
		No.	%	No.	%
Salmonella spp.	4257	36	0,85	4221	99,15
Listeria spp.	2983	21	0,70	2962	99,30
Campylobacter spp.	521	3	0,58	518	99,42
TOTAL	**7761**	**60**	**0,77**	**7701**	**99,23**

Table 6. The results obtained from the examination of the quality indicators by means of rapid diagnostic alternatives

Analized parameters	**Samples No.**	**Results**			
		Noncompliant		**Compliant**	
		No.	%	No.	%
Staphylococ c.p.	855	52	6,08	803	93,93
E. coli	1235	67	5,43	1168	94,57
Enterobacteriaceae	943	79	8,38	864	91,62
NTG	1520	131	8,62	1389	91,38
D + M	570	41	7,19	529	92,81
TOTAL	**5123**	**370**	**7,22**	**4753**	**92,78**

The interpretation was carried out in accordance with the provisions of Regulation 2073/2005 laying down the microbiological safety criteria defining the acceptable process characteristics as well as microbiological safety criteria for foodproducts which set a limit beyond which a food should be considered unacceptable as being contaminated.

It was found that most non-conformities were recorded in case of Totala Number of Germs (8,62%), Enterobacteriaceae (8,38%), *Yeast + Molds* (7,19%), and fewest in case of *Campylobacter spp*.(0,58%), *Listeria spp.*(0,70%) and *Salmonella spp.* (0,85%).

These values express the situation of the analyzed samples from the point of view of microbiological contamination and the obtained results were the basis of the corrective measures implemented in the processing units and of the sanctions imposed on the batches of provenance in the case of identifying the bacterial species with a toxicogenic potential. The original batches were seized until the initial results were confirmed/refused by the reference tests .

The 60 samples with non-compliant results in fast alternative tests for food pathogens were reexamined by standardized reference methods, in accordance with the provisions of

Regulation 2073/2005. A correlation of the 100% obtained results was found as follows: (Table 7)

Table 7. Correlation of results obtained using reference and alternative methods

Analized parameters	**Non-compliant samples**		**Corelation %**
	Alternative methods	**Reference methods**	
Salmonella spp.	36	36	100
Listeria spp.	21	21	100
Campylobacter spp.	3	3	100
TOTAL	60	60	100

The 370 samples with non-compliant results in the rapid alternative tests for quality indicators were examined by standardized reference methods, with a correlation of 98.38% as follows : (Table 8)

Table 8. Conformity of the results obtained using the reference methods

Analized Parameters	**Non-compliant samples**		**Corelation %**
	Alternative methods	**Reference methods**	
Staphylococ c.p.	52	52	100
E. coli	67	67	100
Enterobacteriaceae	79	79	100
NTG	131	126	96,18
D + M	41	40	97,56
TOTAL	370	364	98,38

It means that the Mini Vidas method used for the detection of food pathogens generates equivalent results (100%) and the TEMPO method, used for the detection of quality indicators, generates results with a correlation degree of 98.38%, with those obtained using the reference methods.

However, it is worth noting that in the case of the non-correlations recorded in the quality indicators, Total Number of Germs and Y + M, where the differences were 3.82% and 2.44% respectively, the non-conforming samples insignificantly exceeded the upper limit of the admissibility. This demonstrates that for the TEMPO method, some parameters may have some mismatches when the results record values that are at the limit of compliance / nonconformity.

As far as the bacterial identification is concerned using the Vitek 2 Compact equipment, the 12 samples of *Salmonella spp*. and the 9 samples of *Listeria spp*. were also analized with the Kaufmann-White method.

For both genres, a correlation of 91.68% was found, with significant differences between species Salmonella and Listeria. Thus for the Salmonella genus, the correlation of the results was 77.8, and in the case of the Listeria genus, the correlation of the results was 100%, as follows: (Table 9)

Table 9. Identified serovars *of Salmonella enterica* and *Listeria spp*.

Microorganisms	Species/ Serovar	Vitek 2 Compact Method	Kaufmann-White Method	Correspondence %
Salmonella enterica	*enteritidis*	3	2	66,7
	taksony	1	1	100
	infantis	3	4	66.7
	newport	3	3	100
	saintpaul	2	2	100
Listeria spp.	*L. monocytogenes*	3	3	100
	L. ivanovii	3	3	100
	L. innocua	3	3	100

The non-conformities of the results obtained using the Vitek 2 Compact and Kaufmann-White methods were found in the case of *Salmonella enetritidis* and *Salmonella infantis.* In the case of *Listeria monocytogenes* species, the correlation of the results was 100%.

4. Conclusion

Following the use of alternative diagnostic methods for the examination of food samples taken from processing units, it was found that most of the non-conformities were recorded in the testing of quality indicators and significantly less in the testing of food pathogens.

The obtained results were the basis of the corrective measures implemented in the processing units and the sanctions imposed on the batches of provenance after they were confirmed / refuted by standardized reference methods, according to the provisions of Regulation 2073/2005.

Rapid alternative diagnostic methods used for the detection of food pathogens generated results equivalent to those of the standardized reference methods. In the case of fast alternative diagnostic methods for the detection of quality indicators, the results showed a correlation of 98.38% with those of the standardized reference methods.

The results obtained in bacterial identification, made using alternative and reference methods, such as Vitek 2 Compact and Kaufmann - White, may show some differences.

5. Competing interest

The authors declare no conflict of interest.

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