Romanian Biotechnological Letters *Vol. x, No. x, 2018*

ORIGINAL PAPER

RESEARCH ON THE EFFICIENCY OF THE USE OF ALTERNATIVE DIAGNOSTIC METHODS IN MICROBIOLOGICAL ANALYSIS OF FOOD

Received for publication, August, 3, 2017
Accepted, May, 2, 2018

PUCHIANU GHEORGHE[1], BABII MIHAELA[3], NECULA VALENTIN[1], ENACHE DORIN VALTER[1] , DANEŞ MIHAI[2]

[1] *Transilvania University of Brasov*
[2] *University of Agricultural Sciences and Veterinary Medicine Bucharest*
[3] *Brasov Sanitary-Veterinary and Food Safety Department*
Address for correspondence: Faculty of Food and Tourism, Transilvania University of Brasov, Castelului Street, no. 148, code 500123, Brasov, Tel: 0268472222, Fax 0268472222, email: gpuchianu@yahoo.com

Abstract

In order to verify the effectiveness of the use of alternative diagnostic methods, a total of 9952 samples was collected from the Brasov Sanitary-Veterinary and Food Safety Laboratory, taken from processing units within the county of Braşov.

The examinations were: enumeration of the quality indicators - using the TEMPO equipment; detection of food pathogens - using VIDAS equipment and bacterial identification - using the VITEK 2 COMPACT equipment.

It was found that 4.3% of the examined samples had nonconformities, most of them being recorded in the total number of Germs (8.9%), *Enterobacteriaceae* (8.6%) and *Staphylococcus spp.* , and the least for *Salmonella spp*. (0.9%) *Listeria spp*. (1.8%) and *E. coli* (0.0%).

In the case of samples with non-conforming results in the alternative tests, standardized methods were performed, establishing a 100% correlation level for all parameters analysed. Also, some of the species identified using the Vitek 2 Compact equipment were further identified by the Kaufmann-White method. Species of *Salmonella spp*. (*Salmonella enterica* serovars: *saintpaul, infantis, newport, enteritidis* and *taksony*) and *Listeria spp.* (*L. monocytogenes, L. ivanovii* and *L. innocua*), identified by the Vitek 2 Compact method, were confirmed in 83.3% of the cases of *Salmonella spp*. and in 100% of the cases of *Listeria spp. Infantis* serovar identified by the Vitek 2 Compact method was confirmed by the Kaufmann-White method, as being *taksony*.

Keywords: rapid alternative diagnostic method, standardized method, pathogenic germs.

1. Introduction

Decisions on food safety involve consideration of a wide range of concerns, including the public health impact of foodborne illness, the economic importance of the agricultural sector and the food industry, and the effectiveness and efficiency of interventions. (4)

The presence of microorganisms is particularly important for the quality, wholesomeness and freshness of foodstuffs. Generally the microorganisms are those that reduce the nutritional value of the product, or can be eligible by their pathogenic action, for the degradation and production of toxic metabolites. (8, 9, 14)

Microbiological criteria are very important; they provide guidance on what concerns the acceptability of food and manufacturing processes, manipulation and distribution. For this reason they must be part of the procedure, based on HACCP principles and other measures for the hygiene control, by establishing a limit above which, a food product should be considered unacceptable contaminated. (12)

On food security, an important component of the field as a whole is to achieve food security by the sector operators, in the self-prepared control programs elaborated in accordance

with applicable laws, in which they are obliged to survey all relevant parameters, having in view the specific activity of each unit.

In this respect, the European recent regulations, reunited in the so-called "hygiene package", aimed at preventing random food risks with the obligation to ensure the food safety circuit "from fork to plate", placing all responsibility to the producers, processors and suppliers of food resources, able to bring under qualified control the quality and food health. (11, 13, 14)

Automation in enumeration methods can be very useful to reduce the time needed for the preparation of the culture media, serial dilution, counting colonies, etc. Many improvements in this field have been made, that allow laboratories to increase the efficiency and the number of samples examined, such as agar preparation machines, automated dilutors, automated counting devices and spiral plate. (6, 14)

An ideal detection system should include high specificity and sensitivity; fast response time; capability for mass production; elimination or simplification of the sample preparation steps; minimal perturbation of the sample; and providing continuous data analysis. Much progress has been made for the last decades, including automation and high throughput for sample processing and testing. (3, 7)

Lately, more and more rapid tests for microbiological food expertise are being used. In this regard, we can recall the tests based on the detection of antibodies and nucleic acid that revolutionized the methodology for the detection of microbial pathogens and their toxins. (3)

Many years ago, it has been predicted that traditional methods of microbiological examination will be replaced by automated, rapid methods. (10)

Rapid early detection of food contamination is therefore relevant for the containment of food-borne pathogens. Conventional pathogen detection methods, such as microbiological and biochemical identification, are time-consuming and laborious, while immunological or nucleic acid-based techniques require extensive sample preparation and are not amenable to miniaturization for on-site detection. (1, 5)

It should be noted that the results of the rapid diagnosis methods (which can be used in accordance with the provisions of Regulation 2073/2005) should be confirmed using standardized diagnostic methodologies. (15, 16)

2. Material and Methods

In order to verify the effectiveness of the use of fast alternative diagnostic methods, a total of 9952 samples were examined within the Sanitary Veterinary and Food Safety Laboratory Brasov, taken in the framework of the self-control program in processing units within the county of Brasov.

The microbiological tests carried out were as follows: enumeration of the quality indicators - using the TEMPO equipment; detection of food pathogens - using VIDAS equipment and bacterial identification - using the VITEK 2 COMPACT equipment (Table 1).

Table 1. Number of samples taken and examined by alternative diagnostic techniques

Analyzed parameters	**Collected samples**		**The rapid alternative method used for diagnostic**	
	No.	**%**	**VIDAS**	**TEMPO**
Salmonella spp.	4160	41.8	X	

Listeria spp.	1138	11.4	X	
Staphylococcus c.p.	624	6.3		X
Staphylococcal enterotoxin	52	0.5	X	
Campylobacter spp.	82	0.8	X	
E. coli	1056	10.6		X
E. coli O157	8	0.08	X	
Enterobacteriaceae	864	8.7		X
NTG	1440	14.5		X
D + M	528	5.3		X
TOTAL	**9952**	100		

The Tempo method

It is a fully automated method that allows the quantitative determination of bacteria germs, based on traditional microbiology, based on the multiple tube method. It has a sensitivity and ease of use, allows for a quick result as compared to the classic working method (3-7 days) and time saving in the preparation of media, preparation for glassware sterilization, packaging, labeling, inoculation, plate reading, autoclaving and glassware washing, etc. (Figure 1).

Figure 1. Reading station: Reading, interpreting, validation and transfer of results.

Bacteria germs, the matrices from which they can be identified and the time required for laboratory diagnosis are: (Table 2)

Table 2. Bacteria germs, the matrices from which they can be identified and the time required for laboratory diagnosis.

Microorganisms	**Matrix**	**Required Time (number of hours)**
E. coli	Meat products, mechanically separated meat, cheeses made from thermized milk, products of non-animal origin.	24

Staphylococcus aureus	Cheeses made from thermized raw milk at a lower temperature than pasteurization and thermized milk, milk powder, fish products.	24
Enterobacteriaceae	Bovine, sheep, goat, equine, and pig carcasses, pasteurized milk and pasteurized dairy products, milk powder, ice cream and dairy desserts, infant powder formula and food for medical purposes, egg products.	48
NTG	Raw milk, bovine, sheep, goat, pig, equine, and poultry carcasses, minced and mechanically separated meat.	48
D + M	Bakery products	72

The TEMPO test consists of a card with a transfer tube and an ampule with a specific culture medium. The culture media are desiccated, sterile, ready-to-use, one-time use, selective - TC (Total Coliforms), EC (E. coli), EB (Enterobacteriaceae), STA (coagulase positive Staphylococcus), LAB (Lactic acid bacteria) or non-selective - TVC (NTG), Y + M (Yeast and mold), identified by barcode and color code. (Figure 2) 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-well with three different volumes. The card is then hermetically sealed to avoid any risk of contamination during manipulations. Later, a single stage reading, interpretation, validation, and transfer of the results takes place.

Figure 2. Card with a transfer tube
and an ampoule with a specific culture medium.

Examples:

TVC tempo and EC tempo - During incubation the microorganisms present in the card reduce the substrate from the culture medium and produce a fluorescent signal which is detected by the TEMPO reader. Depending on the number and size of positive wells, the tempo system deducts NTG (for the TVC tempo test) or the number of *E. coli* (for the EC test) present in the initial sample, based on the most probable number calculation.

Tempo TC - The culture medium contains a fluorescent indicator which, when the pH is neutral, emits a signal detected by the tempo reader. During incubation, the total coliforms present in the card ferment lactose from the culture medium resulting in a decrease in pH and the disappearance of the fluorescent signal. Depending on the number and size of the positive wells, the TEMPO system deduces the number of total coliforms present in the initial sample according to the most probable number calculation.

The method that uses the MiniVidas automated analyzer

It is a compact, high-performance system for the identification of highly pathogenic germs (*Salmonella spp., Listeria monocytogenes, Campylobacter jejuni, E. coli O 157, Staphylococcal enterotoxin*), based on the immunological analysis principle.

Its use allows time saving in the preparation of culture media, glassware sterilization, packaging, labeling, inoculation, plate reading, autoclaving, and glassware washing, etc., using ready-to-use reagents.

The MiniVidas Automated Analyzer makes possible a large number of analyzes, safety and ease of use, an automated, standardized, robust device that allows objective reading and delivering a fast result compared to classic working methods. (Figure 3)

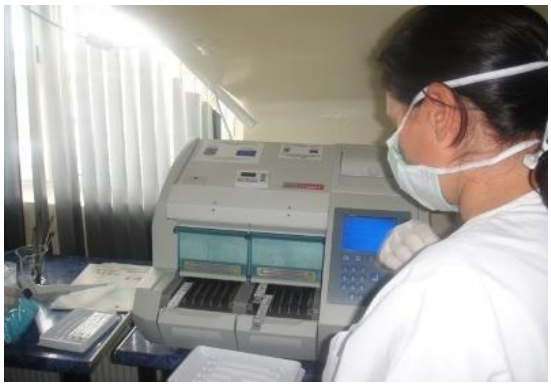

Figure 3. MiniVidas Automated Analyzer

Bacteria germs, the matrices from which they can be identified and the time required for laboratory diagnosis are: (Table 3)

Table 3. Bacteria germs, the matrices from which they can be identified and the time required for laboratory diagnosis

Microorganisms	**Matrix**	**Required Time (number of hours)**
Salmonella spp.	All food products during their shelf life	48
Listeria monocytogenes	All food products before leaving the direct control of the unit which has produced them and products marketed during their shelf life (meat and meat products, raw milk, unpasteurized milk cheeses, confectionery and pastry products, dishes, fish and fish products)	72
Campylobacter jejuni	Poultry carcasses - test to be performed during the warm season (May - September)	72
E. coli O 157	Beef, minced meat and meat products containing bovine meat	72
Staphylococcal enterotoxin	Cheeses made from thermized raw milk at a lower temperature than pasteurization and thermized milk, milk powder, fish products.	72

Vitek 2 Compact

The Vitek 2 Compact equipment is an automatic system for biochemical identification and confirmation, and antibiogram; capable of selecting pathogenic and highly pathogenic organisms isolated on solid media by performing biochemical tests in extremely rapid time, resulting in time saving in generating results.

The use of it saves materials and reagents. Thus, the card used saves all types of reagents needed for identification and confirmation and does not require any type of glassware, thermal sterilization, temperature stabilization or other pre-diagnostic steps.

It's an easy-to-use equipment, with reduced operating time, intuitive software and connection to the LIMS system. The method is fully automated, using different cards to identify Gramm+ (GP), Gramm- (GN), anaerobic bacteria (ANC), Campylobacters (NH), Corynebacteria (CBC), Yeast and Mold (YST), and Bacillus (BCL) in diagnosis.

Identifiable bacteria germs and required time for laboratory diagnosis are as follows: (Table 4)

Table 4. Identifiable bacteria germs and required time for laboratory diagnosis

Identifiable bacteria germs	**Time required for laboratory tests (number of hours)**
E. coli	4-6
Staphylococcus c.p.	4-6
Salmonella spp.	5-6
L.monocytogenes	6-8
Campylobacter jejuni	8-12
E. coli O 157	5-6
Corynebacterium	4-6
Bacillus spp.	4-6
Yeasts and molds, etc.	4-6

The working stages are the following: calibration of the apparatus by using kits specific to 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.

After a variable period of time specified in Table 4, the equipment generates a printed report where the biochemical properties of the identified bacteria genus and the probability of its validation are indicated.

Example: *Salmonella spp.*

3. Results and Discussion

After analyzing the 9952 samples by rapid alternative diagnostic methods, the following results were obtained: (Table 5)

Table 5. Results obtained from examination of samples, using rapid diagnostic alternative techniques.

Analyzed parameters	**Collected samples**		**D.c. Results**			
	No.	**%**	**Non-conforming**		**Conforming**	
			No.	%	No.	%
Salmonella spp.	4160	41.8	36	0.9	4124	99.1
Listeria spp.	1138	11.4	21	1.8	1117	98.2
Staphylococcus aureus	624	6.3	52	8.3	572	91.7
Staphylococcal enterotoxin	52	0.5	4	7.7	48	92.3
Campylobacter spp.	82	0.8	3	3.7	79	96.3
E. coli	1056	10.6	64	6.1	992	93.9

E. coli O157	8	0.08	-	-	8	100
Enterobacteriaceae	864	8.7	74	8.6	790	91.4
NTG	1440	14.5	128	8.9	1312	91.1
D + M	528	5.3	38	7.2	490	92.8
TOTAL	**9952**	100	420	4.3	9532	96.7

It was found that 4.3% of the examined samples tested positive, most of nonconformities being registered for NTG (8.9%), Enterobacteriaceae (8.6%) and *Staphylococcus spp.* (8.3%), and the least for *E. coli* O157, *Salmonella spp.* (0.9%) and *Listeria spp*. (1.8%).

Nonconforming samples from alternative tests were examined by standardized methods as follows (Table 6):

Table 6. Methods used as references

Analyzed parameters	**Number of examined samples**	**Standards used as references**
Salmonella spp.	36	ISO 6579/A1/2017
Listeria spp.	21	ISO 11.290 – 1.2
Staphylococcus spp.	52	ISO 6888 – 1.2
Staphylococcal enterotoxin	4	European screening method of the EU-RL
Campylobacter spp.	3	ISO 16649
E. coli	64	ISO 16649 -1.2
Enterobacteriaceae	74	ISO 21.528 – 1.2
Total Number of Germs	128	ISO 4833/2003
Yeast and Molds	38	ISO 21527-1
Total	**420**	

As for standardized methods, it should be mentioned that their use only in the microbiological food expertise presents some disadvantages: they are laborious; require a longer working time (3-6 days, delaying the delivery of the finished product and providing a delayed response of the data obtained in the hygiene monitoring program); a larger quantity of consumables and numerous suppliers; the results may be subjective (many false negative and false positives), depending on the experience and competence of the people involved in the analytical process and high measurement uncertainty.

However, they remain very important, as they are the standards for the results of other diagnostic methods used in microbiology.

Nonconformities obtained by analyzing samples through alternative methods were 100% confirmed by standardized methods, demonstrating that the alternative methods can be

successfully used, generating results equivalent to those obtained using the reference methods (Table 7).

Table 7. The correlation between alternative methods and standardized methods.

Analyzed parameters	**Alternative methods No.**	**Standardized methods No.**	**Correlation %**
Salmonella spp.	36	36	100
Listeria spp.	21	21	100
Staphylococcus aureus	52	52	100
Staphylococcal Enterotoxin	4	4	100
Campylobacter spp.	3	3	100
E. coli	64	64	100
E. coli O157	-	-	100
Enterobacteriaceae	74	74	100
NTG	128	128	100
D + M	38	38	100
TOTAL	420	420	100

In all cases, the interpretation has been carried out in accordance with the provisions of Regulation 2073/2005, which sets out the microbiological safety criteria defining the acceptability of processes, as well as microbiological safety criteria for food products which set a limit above which a food product must be considered unacceptable as being contaminated.

The obtained values express the situation of the analyzed samples from the microbiological contamination point and the obtained results were the basis of the corrective measures implemented in the processing units and of the sanctions imposed on the consignments of origin in the case of identification of bacterial species with toxicogenic potential. The latter were seized until the results obtained by reference tests were confirmed or refuted.

Some of the species of *Salmonella spp*. (12, representing 33.3%) and *Listeria spp*. (9, representing 42.9%) identified by using the Vitek 2 Compact equipment were further identified by Kaufmann - White method. The analyzes were carried out at the Institute for Hygiene and Veterinary Public Health - Bucharest, with the following results: (Table 8)

Table 8. 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

Species of *Salmonella spp.* (*Salmonella enterica* serovars: *saintpaul, infantis, newport, enteritidis* and *taksony*) and *Listeria spp.* (*L. monocytogenes, L. ivanovii* and *L. Innocua*), identified using the Vitek 2 Compact method, were confirmed in 83.3% of the cases for *Salmonella spp.* and 100% of the cases for *Listeria spp. Infantis* serovar identified using the Vitek 2 Compact method was confirmed using the Kaufmann-White method, as being *taksony*.

It should be noted that of all isolated serovars only *S. enterica* serovar *Enteritidis* and *Listeria monocytogenes* are considered to have a high degree of pathogenicity, being able to cause food poisoning to consumers, while others are of low pathogenicity, requiring assisted heat treatments (under veterinary sanitary surveillance) of foodstuffs, without constituting a risk of illness to consumers.

4. Conclusions

Correspondence between the results of alternative diagnostic and reference methods was at the rate of 100%, demonstrating that alternative diagnostic methods can be used as effective work methods, provided they are developed and validated to adapt to diagnostic conditions specific to each laboratory.

Part of the serovars identified by the Vitek 2 Compact method (*Salmonella spp.* and *Listeria spp*.) were confirmed by the Kaufmann-White method in 83.3% of cases for *Salmonella spp.* and 100% for *Listeria spp.* Pathogenic serovars of *Salmonella spp.* and *Listeria spp.* were confirmed at the rate of 100%.

The results obtained were the basis of the corrective measures implemented in the processing units and the sanctions imposed on the consignments of origin in the case of identifying some pathogenic bacterial species. They were seized until the results obtained by reference tests were confirmed or refuted.

We recommend that rapid diagnostic methods be used in particular to carry out the self-control program of the units, taking into account the limited time for analysis bulletins to be generated in order to make the most efficient use of the results of microbiological examination of foodstuffs.

References

1. AMIT SINGH, SOMAYYEH POSHTIBAN AND STEPHANE EVOY, (2013). Recent Advances in Bacteriophage Based Biosensors for Food-Borne Pathogen Detection. Sensors 2013, 13 (2), pp.1763-1786.
2. Bacterial Nomenclature up - to - date (Approved lists, validation lists), (2012). Compiled by Leibniz Institute DSMZ - Deutsche Sammlung von und Mikroornismen Zellkulturen GmbH, Braunschweig, Germany.
3. BEILEI G, JIANGHONG MENG, (2009). Advanced Technologies for Pathogen and Toxin Detection in Foods: Current Applications and Future Directions. *SLAS TECHNOLOGY: Translating Life Sciences Innovation*. Vol 14, Issue 4, pp. 235 - 241.

4. A. H. HAVELAAR , J. BRÄUNIG, K. CHRISTIANSEN , M. CORNU, T. HALD , M.-J. J. MANGEN, , K. MØLBAK, A. PIELAAT , E. SNARY , W. VAN PELT , A. VELTHUIS AND H. WAHLSTRÖM, (2007). Towards an Integrated Approach in Supporting Microbiological Food Safety Decisions, (2007). Journal compilation, Blackwell Verlag, Berlin, Zoonoses Public Health. 54, pp.103–117.
5. L. JACXSENS, P.A. LUNING, W.J. MARCELIS, T. VAN BOEKEL, J. ROVIRA, S. OSES, M. KOUSTA, E. DROSINOS, V. JASSON, M. UYTTENDAELE, (2011). Tools for the performance assessment and improvement of food safety management systems. Trends in Food Science & Technology. Volume 22, Supplement 1, November 2011, pp. S80 - S89.
6. JAYASHREE ARCOT, ASHOK SHERESTHA, (2005). Folate: methods of analysis in foods. Trends in Food Science & Technology, xx (xxxx) 1- 14.
7. A. JUSTE, B.P.H.J. THOMMA, B. LIEVENS, (2009). Recent advances in molecular techniques to study microbial communities in food-associated matrices and processes. Food Microbiology 25 (2008) 745– 761, www.elsevier.com/locate/fm.
8. PUCHIANU G., BOGDAN A.T., ANDRONIE V., TOBĂ G.F., PETRIU LĂCRIMIOARA, (2011) - Updates of the Veterinary Medicine Role in the Expertise of Food Safety and Security in the World Professional Year, Romanian Academy of Sciences Publishing House, Vol., Nr. 2, ISSN 2068-4096, pp. 409-420.
9. PUCHIANU G, NECULA V., ENACHE D. V., ŞERBAN D., (2014). Research on the efficiency of alternative methods in microbiological expertise of food products. International Conference on New Research in Food and Tourism, Bioatlas, Brasov 15th - 17th May 2014.
10. PURNENDU C. VASAVADA, (1993). Rapid methods and automation in food microbiology: beyond Delphi forecast. Journal of Rapid methods and Automation in Microbiology. Volume 2, Issue 1 March 1993, pp. 1–7.
11. P.-J. RAUGEL, (1999) Rapid Food Analysis and Hygiene Monitoring. Kits, Instruments and Systems. Springer, ISBN 978-3-642-63552-6. 53 -85.
12. ENACHE D., ŞERBAN D., PUCHIANU G., NECULA V., (2014). Ensuring the protection of critical infrastructure in the food industry through the implementation of the „food – defense" – IDESPERAS2L182. The International Conference – Economic Scientific Research – Theoretical, Empirical and Practical Approaches, ESPERA 2014.
13. TERRY A. ROBERTS, (1997). Maximizing the Usefulness of Food Microbiology Research. Emerg Infect Dis. 3(4):523-528.
14. Maximizing the Usefulness of Food Microbiology Research. Emerging Infectious Diseases. Vol. 3, No. 4, October–December 199;
15. VICKY JASSON, LIESBETH JACXSENS, PIETERNEL LUNING, ANDREJA RAJKOVIC, MIEKE UYTTENDAELE, (2010). Alternative microbial methods: An overview and selection criteria. Food Microbiology, 27, 710-730, www.elsevier.com/locate/fm.
16. Council Regulation (EC) 2073/2005 regarding microbiological criteria for foodstuffs published in the Official Journal of the European Union.
17. Council Regulation (EC) Commission Regulation (EC) No 1441/2007 of 5 December 2007 amending Regulation (EC) 2073/2005 on microbiological criteria for food.