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RAPID DETECTION OF *SALMONELLA* IN FOOD BY REDOX-POTENTIAL MEASUREMENT BASED METHOD COMBINED WITH REAL-TIME PCR

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The classical ISO (2002) standard as reference method and the combination of redox potential measurement with real-time PCR technique were applied to detect *Salmonella* in milk, egg, broiler meat, and artificially contaminated egg samples. Food samples of 25 g were homogenized in 225 ml of RVS broth to prepare the basic suspension of the comparative tests. In the combined method the redox potential measurement technique serves as the selective enrichment system of the real-time PCR equipment. The reliable screening of *Salmonella*-free, negative samples by the redox potential measurement technique needed only 24 h. These negative samples determined by the PCR and the classical standard method in all cases proved to be negative as well. In case of positive redox result the *Salmonella* from the enriched suspension of the redox test-cell was identified by real-time PCR in 3 hours, instead of the conventional biochemical identification. Comparing our protocol to the ISO (2002) standard method, the total detection time of *Salmonella* presence/absence was less than 24 h contrary to the 114 h of the conventional method.

Keywords: *Salmonella,* rapid detection, redox potential, real-time PCR, food safety

Salmonellosis is one of the major foodborne diseases, due to its endemic nature, high morbidity and association with a wide range of foods (de Freitas et al., 2010). Foodborne salmonellosis is most often associated with consumption of undercooked beef, poultry, and eggs (DuPont, 2007). In 2010 egg and egg products were the most frequently identified food vehicles causing 43.7% of the strong evidence *Salmonella* outbreaks in the EU, the other most common implicated food vehicle categories were bakery products and broiler meat and products (EFSA, 2012).

Foodstuffs can be contaminated by several *Salmonella* serotypes. *Salmonella enterica* serotype Enteritidis is the predominant serotype associated with egg-borne salmonellosis in humans (Gantois et al., 2008).

The *Salmonella* related food safety criterion, that *Salmonella* may not be present in 25 g products placed on the market during their shelf-life, is laid down in the legislation of most countries. Conventional culture methods used for the detection of *Salmonella* include non-selective pre-enrichment, followed by enrichment and plating on selective and differential agars. Suspected colonies are confirmed biochemically and serologically, possibly taking up to 7 days to complete the entire procedure (Patel et al., 2006).

Although the sensitivity of many modern detection methods has improved significantly, an enrichment step is still needed to overcome the problems of low pathogen numbers and to limit the risk of detecting dead cells (Omiccioli et al., 2009). The enrichment step is required

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not only to increase the target pathogen concentration in a sample but to resuscitate physiologically stressed and injured cells. Selective enrichment is also necessary to suppress the natural background microorganisms as well as to improve detection efficiency and to avoid false negative results (Kawasaki et al., 2010; Garrido et al., 2013).

Molecular methods, such as the polymerase chain reaction (PCR), can rapidly detect and identify food-borne pathogen microbes (Rijpens & Herman, 2002). Real-time PCR for the specific detection of pathogens in foods is increasingly being used as a rapid and reliable tool for the control of contaminated samples along the food production chain. However, the use of PCR can be limited by cost or sample volume (Malorny et al., 2004).

The sample volume of the PCR technique (some ml) does not allow the direct control of the prescribed food safety requirement: no *Salmonella* in 25 g sample (Commission Regulation, 2005). Before taking sample for the PCR test, the microbial concentration of the sample, similarly to the conventional culture methods, had to be increased by selective enrichment. The necessity of the enrichment depends on the microbial concentration of the sample and is independent of the kind of PCR kit.

After enrichment *Salmonella* can be detected by culturing or PCR method. The culture method is time-consuming, the PCR is very expensive. In order to reduce the cost and time requirement of the rapid and reliable detection and identification of *Salmonella,* a redox potential method was chosen that makes it possible to detect *Salmonella* during the enrichment process. The instrumental procedure based on redox potential measurement was originally developed for rapid determination of viable count of several microorganisms in water, milk, foods, hygienic samples, etc. (Reichart et al., 2007; Erdősi et al., 2012). The aim of the present work was to develop a protocol for rapid detection of *Salmonella* by the combination of the redox potential based and real-time PCR methods in a simple time and cost-effective manner.

1. Materials and methods

1.1. Bacterial strains and samples

The *Salmonella* serotypes originated from the National Food Chain Safety Office, Food and Feed Safety Directorate, Food Microbiology Reference Laboratory in Hungary.

The raw milk, egg, and broiler meat samples were obtained from the local market.

1.2. Conventional cultural method

All of the samples were examined according to ISO (2002) conventional cultural method, independently of the parallel applied combination of redox potential based method and real-time PCR.

1.3. Redox potential measuring method

Rappaport-Vassiliadis *Salmonella* selective broth (RVS broth) MERCK 107666 was used as culture medium at the incubation temperature of 42 °C.

The principle of the method (Reichart et al., 2007) is that in the measuring cell due to microbial multiplication, above a quite large (10^6–10^7 CFU ml^{-1}) cell concentration, the redox potential of the medium is well-detectably reduced and the rate of decrease overcomes a defined detection criterion (DC). The time to detection (TTD) means the time necessary to reach the detection criterion. There is a close linear correlation between TTD and the logarithm of the initial viable count, which is represented by the calibration curve.

The determinations were carried out by a 32-channel redox potential measuring instrument, MICROTESTER. The preparation of samples and the instrumental measurements were performed according to the aseptic microbiological routine. The samples were pipetted into the sterile test cells. After closing with the caps containing the sterile electrodes, the test cells were immersed into a water bath of 42 °C. During the measurement the equipment automatically records the redox curves and determines the detection times indicating the probable presence of *Salmonella*. In case of obtaining a detection time, the microbial concentration in the measuring cell is higher than 10^6 CFU ml^{-1} and the medium is ready for direct PCR test.

For the determination of calibration curve, 1 slant agar of the *Salmonella* strain was washed down and a tenfold dilution series was prepared with peptone water to the 6th dilution level. From each dilution 1 ml was pipetted into a redox measuring cell containing 10 ml RVS broth. Detection criterion was set at –0.8 mV min^{-1}. Starting the measurement, the equipment automatically determined the detection times belonging to the several dilution levels. After inputting the viable count of the undiluted inoculum (determined by plate counting), the software computed the calibration curve.

1.4. Real-time PCR

1.4.1. DNA isolation. Genomic DNA was isolated from 1 ml of enriched food sample taken from the redox potential measuring cell at the end of the enrichment process. "*Mericon* DNA Bacteria Kit" (Qiagen) was used according to the manufacturer's instructions.

1.4.2. Real-time PCR assay. Real-time PCR amplification was performed on SLAN® Real-Time PCR System (Hongshi) using *Mericon Salmonella* Kit (Qiagen). The PCR was made with a final volume of 20 µl including 10.4 µl Multiplex PCR Master Mix and 9.6 µl DNA. The assay can detect as few as 10 copies of *Salmonella* DNA in a reaction and it exhibits high specificity.

The cycling parameters consisted of an initial PCR activation step, activation of HotStarTaq *Plus* DNA Polymerase (5 min, 95 °C), followed by 40×3-step-cycles consisting of denaturation (15 sec at 95 °C), annealing for 23 sec at 60 °C, and extension for 10 sec at 72 °C. Fluorescence detection was performed at the end of the annealing stage of each cycle.

Detection of pathogens using real-time PCR was based on the amplification of a specific region of the relevant pathogen genome. The amplified product was detected by using target-specific fluorescent probes. As the PCR product accumulated, there was an increased fluorescent signal from the bound probes. Monitoring the fluorescence intensities during the PCR run (i.e., in real time) allowed the detection of the accumulating PCR product without the need to re-open the reaction tubes afterward. The isolated pure bacterial DNA was assayed using a streamlined real-time PCR protocol.

1.4.3. Preparation of samples. Milk and broiler breast: 25 g sample homogenized in 225 ml RVS broth.

Egg: before opening, the egg-shells were disinfected by washing with 70% ethanol. For the preliminary tests one sample contained the homogeneous mixture of 5 eggs. In case of artificial contamination the basic suspension was the homogeneous mixture of 10 eggs/ contamination level.

For the measurement 25 g sample was homogenized in 225 ml RVS broth.

2. Results and discussion

2.1. Determination of calibration curves of Salmonella *serotypes*

Redox calibration curves of 16 *Salmonella* serotypes were determined in RVS broth in order to calculate the time requirement of the *Salmonella* detection. Based on their calibration curves the strains could be classified in 4 groups, within each group a common calibration curve can be used.

I. *S*. Bredeney, *S*. Kottbus, *S*. Senftenberg, *S.* Stanley, *S*. Tennessee*
II. *S*. Kentucky, *S*. Montevideo, *S*. Ohio, *S*. Saintpaul, *S*. Thompson, *S*. Typhimurium*
III. *S*. Cerro, *S*. Enteritidis*, *S.* Infantis, *S*. Livingstone
IV. *S*. Newport*

The calibration curves of the typical representatives of the groups (signed with * above) are demonstrated in Fig 1.

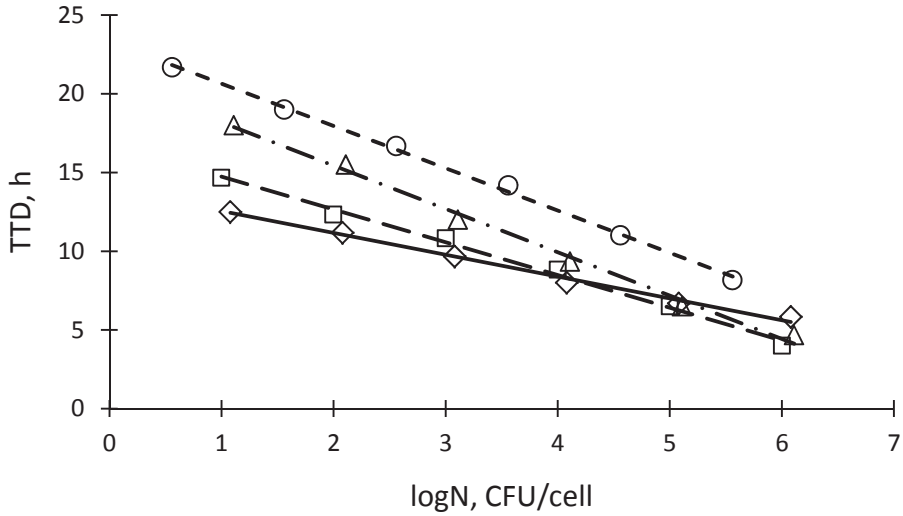

Fig. 1. Calibration curves of *Salmonella* serotypes in RVS, T=42 °C
(◇: *S*. Tennessee; □: *S.* Typhimurium; △: *S*. Enteritidis; ○: *S*. Newport)

The results of regression analysis are summarized in Table 1.

Table 1. Regression of *Salmonella* calibration curves

Microorganism	Data	Equation	R^2	SD (h)
I. *S*. Tennessee	6	TTD (h)= –1,386·logN+13.93	0.9926	0.250
II. *S*. Typhimurium	6	TTD (h)= –2.081·logN+16.81	0.9947	0.319
III. *S*. Enteritidis	6	TTD (h)= –2.752·logN+20.93	0.9938	0.453
IV. *S*. Newport	6	TTD (h)= –2.686·logN+23.33	0.9976	0.278

The time for detecting one target microorganism in the measuring cell can be determined from the intercept of the calibration curve (logN=0). The highest intercept, 23.3 h belongs to *S.* Newport. Taking into account the standard error of the TTD values (0.278 h), the maximum time of detection is about 24 h. When in this period no TTD could be obtained, the sample quantity in the measuring cell was probably free of the target microorganism.

2.2. Preliminary examinations of food samples

Salmonella contamination (present/absent test) of milk, whole egg, and broiler breast meat samples was determined. From each food type 5–5 samples were examined in 2 parallels by conventional method, redox potential method, and real-time PCR following the redox potential measurement (30 determinations per method). The results obtained by the three methods were the same.

Milk and egg samples proved to be *Salmonella* negative. From the 5 meat samples only 1 was negative, while the other 4 samples contained *Salmonella*. Based on these results, in the comparative study of the *Salmonella* test methods, artificial contamination was applied in case of egg samples, while the meat samples were investigated in the original state.

2.3. Salmonella *detection in artificially contaminated egg*

Twenty-five grams of basic suspension was homogenized in 225 ml RVS broth and was inoculated with 1 ml diluted suspension of *Salmonella* Enteritidis and *Salmonella* Typhimurium, separately (24 h slant Plate Count Agar culture washed down and diluted with peptone water). Three contamination levels (low, medium, and high) were set for the microbes. The actual microbial counts are summarized in Table 2.

Table 2. Artificial microbial contamination level of egg samples (CFU/25g)

Contamination	Low	Medium	High
S. Typhimurium	$1.6 \cdot 10^0$	$2.4 \cdot 10^2$	$3.6 \cdot 10^4$
S. Enteritidis	$1.4 \cdot 10^0$	$3.2 \cdot 10^2$	$2.4 \cdot 10^4$

Salmonella contamination was tested in 3 parallels per method. The time requirement of detection by the redox potential measurement was maximum 24 h, but in highly contaminated samples this time significantly decreased. The identification from the enriched suspension by real-time PCR required further 3 h. The results are summarized in Table 3. The 3 methods gave identical results in all cases (21 determinations per method).

Table 3. Detection of *Salmonella* in egg. Mean of 3 parallels

Method	Conventional		Redox potential		Real-time PCR		Redox+PCR
	result	time (h)	result	time(h)	result	time (h)	time (h)
Basic level	–	114	–	24*	–	3**	27
Low level of							
S. Enteritidis	+	114	+	20.6[a]	+	3	23.6
S. Typhimurium	+	114	+	16.2[b]	+	3	19.2
Middle level of							
S. Enteritidis	+	114	+	16.7[a]	+	3	19.7
S. Typhimurium	+	114	+	11.7[b]	+	3	14.7
High level of							
S. Enteritidis	+	114	+	13.2[a]	+	3	16.2
S. Typhimurium	+	114	+	8.7[b]	+	3	11.7
Determinations	21		21		21		21

*: no redox curve; **: no signal; [a]: SD=0.45 h, from Table 1; [b]: SD=0.32 h, from Table 1

2.4. Salmonella *detection in broiler meat*

Salmonella contamination of 20 meat samples was tested in 3 parallels per method. All of the samples were examined also by the ISO (2002) conventional cultural method, parallel with the combination of redox potential based method and real-time PCR. The results are summarized in Table 4. The 3 methods gave identical results in all cases (60 determinations per method).

3. Conclusions

During the comparative study on detection (presence/absence test) of *Salmonella,* milk, egg and broiler meat samples were investigated. Both in case of positive (18 egg- and 65 meat-investigations) and negative (10 milk-, 13 egg-, and 5 meat-investigations) samples the three methods gave identical results.

In the combined method the redox potential measurement technique serves as the selective enrichment system of the real-time PCR equipment. The reliable screening of *Salmonella*-free, negative samples by the redox potential measurement technique needed only 24 h. These negative samples determined by the PCR and the classical standard method in all cases (28 investigations) proved to be negative as well. It concludes that in case of negative redox result the PCR test could be omitted.

Table 4. Detection of *Salmonella* in broiler meat. Mean of 3 parallel detection times (h)

Sample	Conventional		Redox potential method			Real-Time PCR		Total[a]
	result	time	result	time	SD (h)	result	time	time
1.	+	114	+	11.7	0.123	+	3	14.7
2.	+	114	+	10.2	0.423	+	3	13.2
3.	+	114	+	15.8	0.113	+	3	18.8
4.	+	114	+	14.3	0.192	+	3	17.3
5.	+	114	+	13.6	0.280	+	3	16.6
6.	+	114	+	11.6	0.260	+	3	14.6
7.	+	114	+	9.2	0.323	+	3	12.2
8.	+	114	+	15.0	0.413	+	3	18.0
9.	–	66	–	24*	*	–	3**	27
10.	+	114	+	11.0	0.390	+	3	14.0
11.	+	114	+	11.8	0.303	+	3	14.8
12.	+	114	+	11.5	0.250	+	3	14.5
13.	+	114	+	13.0	0.250	+	3	16.0
14.	+	114	+	13.7	0.123	+	3	16.7
15.	+	114	+	12.3	0.333	+	3	15.3
16.	+	114	+	10.3	0.333	+	3	13.3
17.	+	114	+	8.8	0.250	+	3	11.8
18.	+	114	+	8.8	0.178	+	3	11.8
19.	+	114	+	15.2	0.373	+	3	18.2
20.	+	114	+	10.3	0.161	+	3	13.3
Determination	60		60			60		60

[a]: Total=Redox + PCR method; * : no redox curve; **: no signal

In case of positive redox result the *Salmonella* from the enriched suspension of the redox test-cell was identified by real-time PCR in 3 hours, instead of the conventional biochemical identification. Comparing our protocol to the ISO (2002) standard method, the total detection time of *Salmonella* presence/absence was less than 24 h contrary to the 114 h of the conventional method. The combination of the redox potential and PCR methods is labour-saving and it significantly decreases the cost and time of the detection of *Salmonella*.

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