Faculty of Veterinary Science, Szent István University
Food Hygiene Department

# Detection of *Salmonella* Enteritidis and Typhimurium in fresh poultry meat by the combination of redox potential measurement and real-time PCR methods

Gabriella Sjöblom

Tutor: Dr. Erdősi Orsolya
Dr. Szakmár Katalin
Food hygiene department

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# 1. Introduction

Salmonellosis is a zoonotic disease and one of the most distributed food-borne illnesses worldwide causing tens of millions of human cases each year (Radhika et al., 2014). In the European Union an estimated number of 100,000 cases are reported annually. It cannot be eradicated completely but a continuous effort to reduce its occurrence in all steps of the production chain, from farm to fork is necessary for the reduction in animals, food and humans (SVA, 2014). The EU has implemented an "integrated approach to food safety" that involves risk assessment, risk management and the communication between EU member states, European Commission, European Parliament, European Food Safety Association (EFSA) and the European Centre for Disease Prevention and Control (ECDC). Between 2004 and 2009 the integrated approach managed to decrease the number of human salmonellosis by almost 50%. One of the main targets has been to reduce the incidence of *salmonella* in poultry (broilers, breeding/laying hens and turkey). Restriction of trade of infected poultry flocks between countries and providing scientific advice from the European Food Safety Association are some of the methods used to set this goal into action.

In regulation EC 2073/2005 the food safety criteria states that *Salmonella* Enteritidis and Typhimurium have to be completely absent in 25 g of fresh poultry meat placed on the market during their shelf-life to ensure public health (Council Regulation 2073/2005). There are various detection methods used in food microbiology for establishing the safety of foodstuffs. The combination of non-selective pre-enrichment with enrichment and plating on selective and differential agars is traditionally used followed by serological and biochemical techniques for verification. These procedures have shown to be valuable due to accuracy of results, however, a downfall being time-consuming taking up to seven days for results to be obtained (Patel et al., 2006). The delayed diagnosis time is a disadvantage for patients waiting for results, postponing their treatment and being a risk for infecting others (Cunningham et al., 2011).

An enrichment step is required for the modern molecular techniques. These methods are sensitive and there is a risk of detecting dead cells leading to false positive results. The enrichment ensures there are adequate viable pathogen numbers by resuscitating physiologically stressed and injured cells (Omiccioli et al., 2009). To avoid false negative

results, a selective enrichment is prepared to inhibit the growth of non-desirable microorganisms and to enhance the efficiency of determination of the investigated pathogens (Garrido et al., 2013; Kawasaki et al., 2010). For the rapid detection of contamination of foodstuffs molecular methods such as polymerase chain reaction (PCR) can be used for the identification (Riipens & Herman, 2002). For the analysis of contaminated samples real-time PCR has demonstrated to be a reliable tool although disadvantages such as cost and sample quantity can limit its use (Malorney et al., 2004).

In this study, a redox potential method was combined with real-time PCR to reduce costs and time needed for detection. The possibility of identifying *Salmonella* throughout the enrichment phase makes the reduction in cost and time achievable. The redox potential method was initially developed for the determination of microorganisms in water, milk, hygienic samples and foodstuffs (Reichart et al, 2006; Erdösi et al., 2014).

The objective of the study is develop the combination of the redox potential method as enrichment phase and the real-time PCR method as an effective and profitable technique for the detection of *Salmonella* Enteritidis and Typhimurium in fresh poultry meat.

## 2. Survey of literature

### 2.1 Salmonella

*Salmonella* is a gram-negative, facultative anaerobic rod-shaped bacterium belonging to the family Enterobacteriaceae whose optimum temperature is 36-37ºC (survives between 7,0-49,5ºC). The optimum pH is between 7,0-7,5 and water activity 0.94-0,99. Survival in dry environments is characteristic for the *Salmonella* species while freezing does not ensure the inactivation of *Salmonella* species and heat treatment of foods is required at 60ºC for at least 2 minutes or at 70ºC for 1 minute or less (Jozwiak, A., 2013).

The nomenclature of *Salmonella* is complex causing several nomenclatural systems to be used by scientists (Brenner et al., 2000). Unfortunately this may cause confusion due to the inconsistent classification of the genus into species, subspecies, subgenera, groups, subgroups and serotypes (serovars). Kauffman-White instituted the one-serotype one-species concept

based upon the serologic identification of O (somatic) and H (flagellar) antigens. Further methods to categorize species are based on the clinical role of a strain, the biochemical characteristics and on the genomic relatedness (Brenner et al., 2000). Nomenclature schemes used by CDC (Centers for Disease Control and Prevention) and WHO (World Health Organization) recognize only two species in the genus *Salmonella*; *S. enterica* and *S. bongori* (The center for food security and public health, Iowa state university, 2005).
The role of WHOCC-Salm (World Health Organization Centre for reference and research of Salmonella) is to update the *Salmonella* serotyping scheme. The Kauffman-White scheme was first published in 1934 and listed 44 serovars. Between 1965-1989 Le Minor published annual supplements. In 1964 the scheme consisted of 958 serovars. Today we know of at least 2579 serovars and the scheme is now known as the "White-Kauffman-Le Minor scheme" (World Health Organization, 2007).
*Salmonella* is divided into two species; *Salmonella enterica* and *Salmonella bongori* (Iwen, 2014). The majority of the *Salmonella* bacteriae belongs to the former and can be subdivided further into six subspecies: *S. enterica, S. salamae, S. arizonae, S. diarizonae, S. houtenae* and S. *indica,* or I, II, IIIa, IIIb, IV and VI respectively. Subspecies I is associated with disease in warm-blooded animals (Porwollik et al., 2004). The subspecies are further divided into over 50 serogroups based on the somatic (O) antigens present. There are over 2500 serovars (serotypes) based on the flagellar (H) antigens where serovar *Salmonella* Typhimurium and *Salmonella* Enteritidis can be divided into different phage types (Lin-Hui Su et al., 2007).
Serotyping is important for the epidemiological identification of bacteria that is performed by detecting surface antigens (LPS and O-antigens) and flagellar antigens (proteins and H-antigens).

The affirmation of new serovars occurs when the WHOCC-Salm (Institut Pasteur, Paris), Institut für Hygiene und Umwelt (laboratory in Hamburg) and Centers for Disease Control (Atlanta) are in agreement of the validation of the new serovar.

Present number of serovars in each species and subspecies:

***S. enterica*****:** 2 557

*S. enterica subsp. enterica*: 1 531

*S. enterica subsp. salamae*: 505

*S. enterica subsp. arizonae*: 99

*S. enterica subsp. diarizonae*: 336

*S. enterica subsp. houtenae*: 73

*S. enterica subsp. indica*: 13

***S. bongori*****:** 22

**Total**: 2579

The somatic O-antigens where previously categorized by letters but considering there were not enough letters it became necessary to continue with numbers 51 to 67. Today the somatic antigens are labeled with O and the number of the characteristic O-factor and occasionally with the letter in brackets. Underlined factors are certified by phage conversion “which are present only if the culture is lysogenized by the corresponding converting phage” (Grimont & Weill, 2007).

O-antigens expressed in brackets [ ] indicate it may or may not be present without any relation to phage conversion. The brackets in H-antigens demonstrate that they are particularly found in wild strains.

*Salmonella* Typhimurium

Somatic (O): 1,4, [5], 12

Flagellar (H): i (phase 1) and 1,2 (phase 2)

*Salmonella* Enteritidis

Somatic (O): 1, 9, 12

Flagellar (H): g,m (phase 1) antigens and no phase 2 antigens.

## 2.2. Salmonellosis

In 2009 the European Food Safety Authority was requested to provide an estimate of different flock prevalence values of different serovar groups of *Salmonella* (*S*. Typhimurium, *S*. Enteritidis vs. all *Salmonella* serovars with public health significance). The methodology was followed according to Regulation (EC) number 2160/2003. During this year there were 324 *Salmonella* outbreaks; mainly in France, Poland and Spain. Like previous years *Salmonella* Enteritidis accounted for the most number of outbreaks (59,6%) while *Salmonella* Typhimurium accounted for 15,7% of total outbreaks.

Undercooked and raw eggs and poultry meat is one of the highest risk factors of salmonellosis in humans. According to the European Food Safety Authority in 2006, 20,3% of large-scale flocks were positive for *S*. Enteritidis in the EU and in some countries the prevalence was higher than 80% (Malorney et al., 2007).

The infection in humans, animals and foodstuffs is notifiable in the European Union and routes of infection occurs mainly in a fecal-oral route via meat, egg, vegetables, contaminated environment as well as transmission via contact between humans or animals. "In animals *Salmonella spp.* are carried asymptomatically in the intestines or gall bladder and are continuously or intermittently shed in the feces" (The center for food security and public health, Iowa state university, 2005) and therefore may not be detected by mere clinical signs. The disease can also be carried latently in lymph nodes and tonsils and cause outbreak when the animal is immunosuppressed or in stress (SVA, 2014). In some cases *Salmonella* can cause acute gastrointestinal illness ranging from mild to severe symptoms. Fowl can be

infected with several different types of *Salmonella*; *Salmonella* enterica serovar Gallinarum biovar Pullorum and Gallinarum causes pullorum disease and fowl typhoid respectively. These are avian specific and lack motility due to the absence of flagella. *Salmonella* Typhimurium and Enteritidis are not avian specific but may cause persisting infections that may or may not show clinical signs. In the poultry industry the control of *Salmonella* is essential for the health of animals, humans and for the final poultry product. Vertical transmission can occur, causing infection in eggs, externally and internally. Horizontal transmission takes place via bird-to-bird contact, contaminated water, faeces, and litter or by workers and equipment (Soria et al., 2012). In an experiment performed by Garcia et al., 2011 it was shown that contamination via faeces was most common (92%) compared to eggshells (34%) and cloacal swabs.

Human salmonellosis causes symptoms of diarrhoea, abdominal cramps and fever. The incubation time is usually between 1-3 days but can lie in a range between 6 hours and 10 days. Most recover spontaneously while in 1-15% of the affected, prolonged infections can occur, causing septicaemia and reactive arthritis. According to SVA (Statens Veterinärmedicinska Anstalt, 2014), "prolonged symptomless excretion of the pathogen is common". The infective dose is relatively high; a healthy grown-up with a normal functioning immune system needs approximately $10^5$-$10^6$ cells to get infected (Jozwiak, 2013). The infection is usually self-limiting whereby fluid therapy may be required in some patients. Antibiotic treatment is not recommended due to observed ineffective results and prolonged faecal shedding. Most serovars can infect both humans and animals whereby some *Salmonella* types are more specific towards certain animal species; *S*. Derby and S. Choleraesuis in swine, *S*. Dublin in cattle and *S*. Typhi in humans (SVA, 2014). Human Salmonellosis is mostly associated with *Salmonella* Typhimurium and *Salmonella* Enteritidis due to consumption of contaminated eggs and broiler meat. The European regulation 2160/2003 is a directive to control that appropriate measures are taken for the detection and control of *Salmonella* species. Consumers have a responsibility of separating fresh and prepared meat, washing utensils, washings hands, disinfection of areas used and ensuring the meat is not undercooked.

The EU directive 2003/99/EC provides the legal background and controlling of zoonosis and zoonotic agents in the EU. Its purpose is to monitor food-borne outbreaks and ensure proper epidemiological investigations accordingly. *Salmonella* monitoring is referred to Regulation

(EC) 2160/2003 in which all food producers have to agree on having samples taken for testing. In case of broilers, analysis is based on all *Salmonella* strains that may be a health concern for the public and shall cover specifically *Salmonella* Typhimurium and *Salmonella* Enteritidis.

In 2005 a baseline study was put into action in which all member states of the EU participated. In holdings of 5000 birds or more, the flocks were tested three weeks before slaughter for *Salmonella*. Five pairs of boot swabs in each flock were sampled and collected for testing. After evaluation of the results, a target was set to decrease the positive percentage of flocks to less than 1% by December 31, 2011. The reinforcement of National Control Programs (NCP) was set in place in which all broiler flocks of each country must be included. A requirement was set that samples had to be taken three weeks before slaughter and that an official control of sampling at least one flock of broilers on 10% of the holdings with more than 5000 birds.

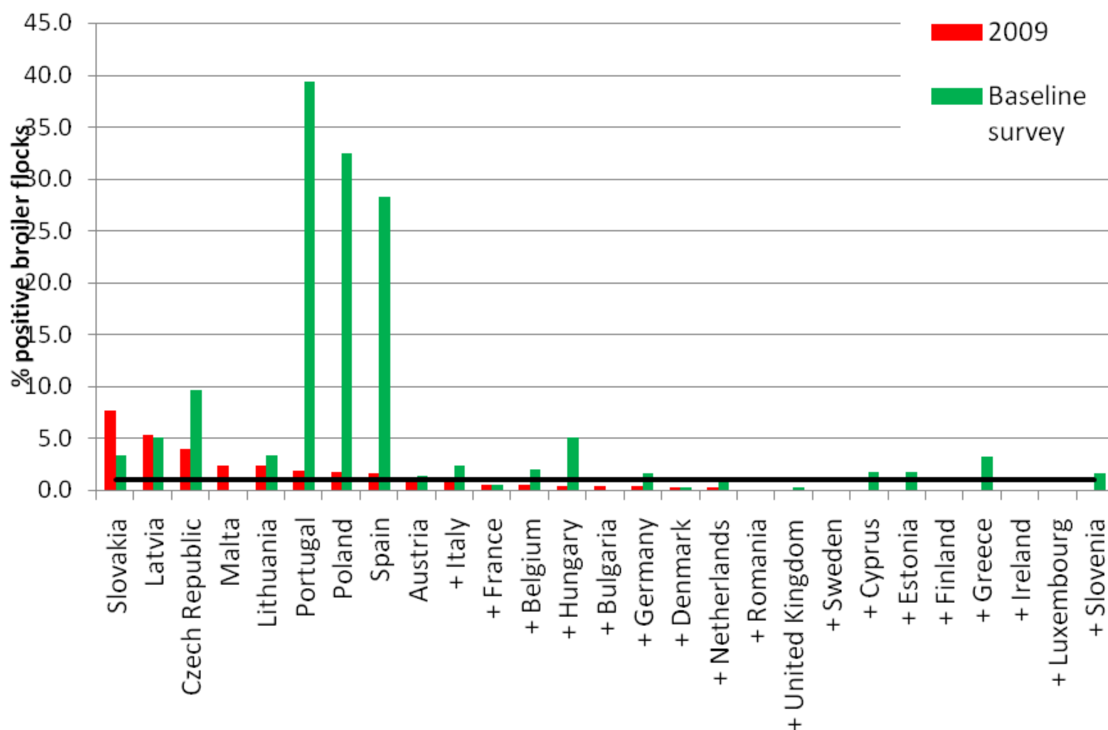


**Figure 1.** Prevalence of *S*. Typhimurium and *S*. Enteritidis in poultry flocks of different member states reported in 2009. Black line represents target for 2010 (+ meaning target already met (EFSA)

The results showed that 18 member states of the European Union were able to set the target of 1% or less in which seven of those states reported no findings of *Salmonella* at all.
In 2008 a baseline study was carried out for the detection of *Campylobacter* and *Salmonella* in broiler carcasses. The sampling was based on random testing of broilers in slaughterhouses, the sampling days in each month and the batches to be sampled. The results concluded that there were some variations between *Salmonella* contamination between broiler flocks and broiler meat in which some member states had positive results in both while in other member states serovars were isolated from broiler carcasses but not from the flock population.

**Table 1.** Reported cases of *S*. Enteritidis and *S*. Typhimurium causing human Salmonellosis in the EU (Based on EFSA and ECDC 2011, 2010 and 2009)

| **Serovar** | **2006 (N)** | **2006 (%)** | **2007 (N)** | **2007 (%)** | **2008 (N)** | **2008 (%)** | **2009 (N)** | **2009 (%)** |
|---|---|---|---|---|---|---|---|---|
| S. Enteritidis | 90,362 | 71 | 81,472 | 64,5 | 70,091 | 58 | 53,382 | 52,3 |
| S. Typhimurium | 18,685 | 14,7 | 20,781 | 16,5 | 26,423 | 21,9 | 23,759 | 23,3 |

The trend of *S*. Enteritidis infections have decreased since 2006 while *S*. Typhimurium cases have increased. An improved control of *S*. Enteritidis in breeding and laying hens is the presumed cause for the decreasing trend since vertical transmission is decreased. Monophasic *S*. Typhimurium-like strains have recently become of greater importance, especially in eggs, pig meat, beef or milk products and are becoming a threat for broiler production as well.

## 2.3 Legislation

In regulation EC 2073/2005 the microbiological requirements of poultry meat and products are described. It provides the basics of food law and is important for ensuring the public health using two types of criteria, which include a food safety criterion, and technological hygiene criterion. It is stated that foodstuffs should not contain microorganisms in quantities

that may cause a risk of health and not be marketed if unsafe. The importance of good hygienic measures is based on the Hazard Analysis and Critical Control Points (HACCP) principles.

The food safety criterion states that a food product which is not satisfactory must either not be marketed or be withdrawn. The supply, handling and processing of raw materials and foodstuffs have to meet the hygienic criteria; food operators at all stages must ensure this is met. The regulation stated that the limit was the absence of *Salmonella* Enteritidis and Typhimurium in 25 g of fresh poultry meat; out of 5 samples 0 could be positive. The analytical reference method for this criterion the EN/ISO 6579 (for detection) White-Kaufmann-Le Minor scheme (for serotyping) should be used.

## 2.4 Salmonella detection methods

### *2.4.1 Conventional (or Standard method)*

Due to its selectivity and sensitivity, the culture method remains the gold standard for the detection of *Salmonella*. The culture method takes on average 5 days in order for the bacteria to proliferate and to form visible colonies that can further be analysed by biochemical and serological tests. Biochemical tests for *Salmonella* include:

- Glucose fermentation: positive
- Lactose fermentation: usually negative
- Sucrose fermentation: negative
- Urease reaction: negative
- Lysine decarboxylase: positive
- Indole test: negative
- $H_2S$ production: usually positive
- Methyl red test: positive
- ONPG test: usually negative

There are four phases needed for the traditional culture method to be carried out. The first phase is the pre-enrichment phase, which enables cells with low viability to be restored and to establish multiplication of the target microbes. This occurs in a non-selective medium. The second phase take place in s selective medium with enrichment for growth and survival of *Salmonella* species while inhibiting the growth of other microbes in the media. In the third phase a selective agar media is used for the isolation of *Salmonella* species and inhibiting the growth of other bacteria on the plate. Lastly, morphological, biochemical and serological tests are performed for confirmation (H. Van der Zee, 2003).

There are various types of media used such as selective enrichment broths and selective agar plates for the determination of *salmonella* contamination of food and food ingredients. The media may contain certain inhibitors to block the growth of other bacteria or consist of substrates that are only degradable by the target bacteria. Selective enrichment broths include Selenite Cystine broth (SC), Rappaport-Vassiliadis Soy broth (RVS), Tetrathionate broth (TT) or Muller-Kauffman Tetrathionate Novobiocin broth (MKTTn) that need to be incubated at 37 or 42 $^{o}$ C for 18-24 hours. Selective agar plate include Xylose Lysine Deoxycholate agar (XLD agar), Bismuth Sulphite agar (BIS), Brilliant Green agar (BG) with or without the addition of sulfadiazine or sulfapyridine (BGS), modified semisolid Rappaport Vassiliadis (MSRV), Salmonella Shigella agar, or Hektoen Enteric agar (Odumeru et al., 2012).
On selective agar plates, *Salmonella* colonies can be detected after 24 hours as whitish-grey medium sized colonies of approximately 2-4 mm.

For serological examinations polyvalent antisera for somatic and flagellar antigens are used. Agglutination of isolates with both somatic and flagellar antisera is classified as *Salmonella* species. Isolates that are positive can be further serotyped into serovars by using specific antisera using the Kauffman-White typing scheme. The time requirement of the method is 2-7 days.

### *2. 4.2 Immunological methods*

Examples of immunological methods of *Salmonella* detection in food include rapid agglutination assays, enzyme-linked immunosorbent assay (ELISA) and lateral flow immunoassays.

The rapid latex agglutination assays are mainly used for the confirmation after presumed *Salmonella* colonies growth on selective agar plates. When an antigen binds to an antibody, these form immunocomplexes that cause agglutination. A colour agglutination against an altered background implies a positive result whereas in a negative result there is no colour change and “the latex remains in a smooth suspension” according to Odumeru. J.A., Leon-Velarde, C.G., (2012).

ELISA is based on the detection of an antigen in a sample. By immobilization of the antigen on a polystyrene microtitre plate and then adding the detection antibody, this forms a complex that can be detected. Dilutions of sera are tested in wells of the microtitre plates coated with the antibody of interest. The sample suspected of containing the antigen is added and if antigens specific to the antibodies are present in the serum, these will bind and form complexes. The wells are then washed to remove the unbound antigens. An enzyme-linked antibody is added which binds to the antigen and is retained. A colourless substrate for the enzyme is added and if there is a colour change, this indicates the presence of an antigen (direct ELISA). In indirect ELISA the presence of an antibody is tested.

Lateral flow immunoassays use a polyclonal antibody as a “capture antibody” and a monoclonal antibody as a “detection antibody” which are based on a series of capillary beds. Each of these has the capability to transport fluid precipitately. Results can be obtained in 24 hours, however, false positive results may occur with this method.

### 2.4.3. Rapid microbial methods

#### *2.4.3.1. Impedimetric method*

The impedimetric method of measuring the presence of microbial contamination has been the preferred technique that emerged in the beginning of the 1970s. It has been valuable for obtaining quantitative and qualitative results in detection of bacteria, yeasts and moulds.
The impedimetric method is based on the theory that there is a change in impedance when microbes in the media start to proliferate (Hayes, P., 1992). This will cause a change in the ionic character of the medium due to the production of highly charged metabolic molecules such as organic acids, fatty acids and amino acids (Amorim et al., 2009). The result is a change in the curve that is known as the time to detection (TTD). The change is inversely proportional to the initial number of microbes. The temperature and media have to remain constant in order for a calibration curve to be established.

The selective media used for impedimetric system usually contains high salt levels (e.g. Lithium Chloride or Magnesium Chloride) due to its high conductance readings. Since these work outside of the normal working range of the impedimetric system, an indirect technique has to be used. The production of $CO_2$ of the microbes can be monitored by the absorbance of the $CO_2$ molecules using a potassium hydroxide agar bridge that will decrease the conductance (Reichart et al., 2006).

There are however several disadvantages with the impedimetric method. Given low concentrations of initial cell count, the linear relationship between the time to detection and the initial number of living cells is not able to be determined. If the initial living cell count is $10^2$cells/ml or less, the impedimetric system is not able to produce accurate result. Interference by competitor organisms that can cause cross-reactions may produce false positive results or “mask” target organisms (Amorim et al, 2009). In case of media with high salt concentrations the indirect method of monitoring the $CO_2$ production has to be used. However, all bacteria do no produce $CO_2$ that makes the detection faulty, unreliable and difficult to use. The impedimetric system requires strict temperature control as the impedance is highly influenced by variations in temperature. The fluctuation may not exceed ±0,002 $^{o}$ C (Reichart et al, 2006).

#### *2.4.3.2. Redox potential*

The oxidation-reduction reaction performed by the microorganisms is the basis of the fast microbiological method. There is a reduction of the medium when the microorganisms use the oxidation as an energy source. The electric effect change can be expressed by the Nernst equation:

$$E_h = E^\circ + RT/nF \ln Q$$

$E_h$ : Redox potential (V)

$E^\circ$ : Normal redox potential of the system (V)

R: Gas constant (8.314 J/mol K)

T: Temperature (K)

F: Faraday constant ($9.648 \cdot 10^4$ C/mol)

n: Number of electrons in the redox system

lnQ: the natural log of the reaction quotient at the moment in time ([oxidant]·$[H^+]$/[reductant])

The shape of the redox potential curve is characteristic of the type of microorganism and the rate of change is proportional to the living cell concentration. The time to detection (TTD) is the time required to reach a considerable change in redox potential and is “proportional to the logarithm of the initial microbial population size” (Reichart, et al., 2006). Between concentrations of $10^0$-$10^7$ cells/ml the correlation between the time to detection and the initial number of living cells is linear.

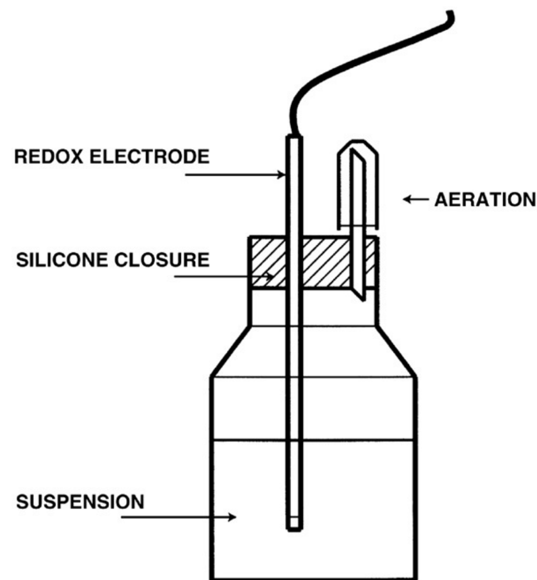


**Fig. 2** Test cell for redox potential measurement (Reichart et al., 2006)

The MicroTester has been developed by the staff of Szent Istvan University, Faculty of Veterinary Science, Department of Food hygiene and the staff from Corvinus University, Faculty of Food Science, Department of Physics and Automation. The MicroTester uses the principle of redox potential to detect quantitatively and qualitatively microbes in a sample.

The MicroTester system is different from impedimetric systems in the way that there is no limit of sample size since the redox potential is not dependent on the shape and volume of the test cell. It is useful in the use of commercial nutrient media, when membrane filtration methods are applied and when surface hygienic samples are measured. With classic microbiological methods the minimum time needed for detection time is at least 24 hours, reaching up to 72 hours in many cases and to make the Hazard Analysis Critical Control Points Systems more effective there has been a need for detection methods to become more rapid and cost as little as possible. The precision of temperature does not have to be as accurate as in the impedimetric system. A change in temperature of 1ºC causes a change in the redox potential between 0,5-1,5 mV that is negligible. A precision of ±0,5ºC is accepted, compared to ±0,002ºC in conductivity methods. The redox potential method does not require the strict temperature control; most nutrient broths can be used and is cheap in comparison to

the impedimetric/conductance methods. The redox potential and impedimetric methods are similar in mode of action but the impedimetric system has shown a paucity of advantages and accuracy in comparison to the redox potential system method.

#### *2.4.3.3. Polymerase Chain Reaction (PCR)*

Polymerase chain reaction or, PCR, is an effective tool used to copy a specific segment of DNA and may be used to diagnose diseases caused by bacteria or viruses. To complete PCR reactions primers, DNA polymerase and nucleotides are needed. Primers are custom made pieces of DNA which has the sequence of nucleotides needed to match the segment of DNA to be copied. Two primers are needed, one which attaches to the top strand of the segment and the other on the bottom strand at the other end. When DNA polymerase bumps into a primer it starts to add nucleotides to the segment to be copied.

Cells are used to extract the DNA, which is moved to a PCR tube that is designated for even heat distribution. Primers are added which attaches to the site of the DNA strand to be copied. Nucleotides (A, T, G and C) are added to the PCR tube that makes up the genetic code. Finally DNA Polymerase enzyme is added to the tube that read the DNA code of the strand to be copied and attach the matching nucleotides to create the DNA copies. The PCR tube is placed into a DNA thermal cycler, which precisely heats and cools the tube at specific time intervals crucial for the reaction to work.

During cycle one the heater heats up to 95$^{o}$C that causes the DNA double helix to separate, forming two single-stranded DNA molecules. The thermal cycler then cools down to 50$^{o}$C causing the single-stranded DNA molecules attempt to pair up. Before this occurs, the primers attaches to their target sites before the strands can re-join.

In the third phase of the thermal cycler, the tube is heated again to 72$^{o}$C, activation the DNA polymerase enzyme occurs. When the enzyme locates a primer, it adds complementary nucleotides to the specific site and continues until reaching the end of the strand and this completes a single cycle. Reaching cycle three, the aimed products appear; two strands that begins with primer 1 and end with primer 2. These are the DNA copies of the targeted segment. Repeating the cycles over and over will cause the solution to contain mainly of the target sequence.

A PCR reaction consists of three phases; an exponential, a linear and a plateau phase as seen in figure 3.

In traditional PCR results are obtained at the end of the reaction, meaning it may take days to collect the results, which makes this method very time consuming. Other disadvantages of end-time PCR are poor precision and low sensitivity.

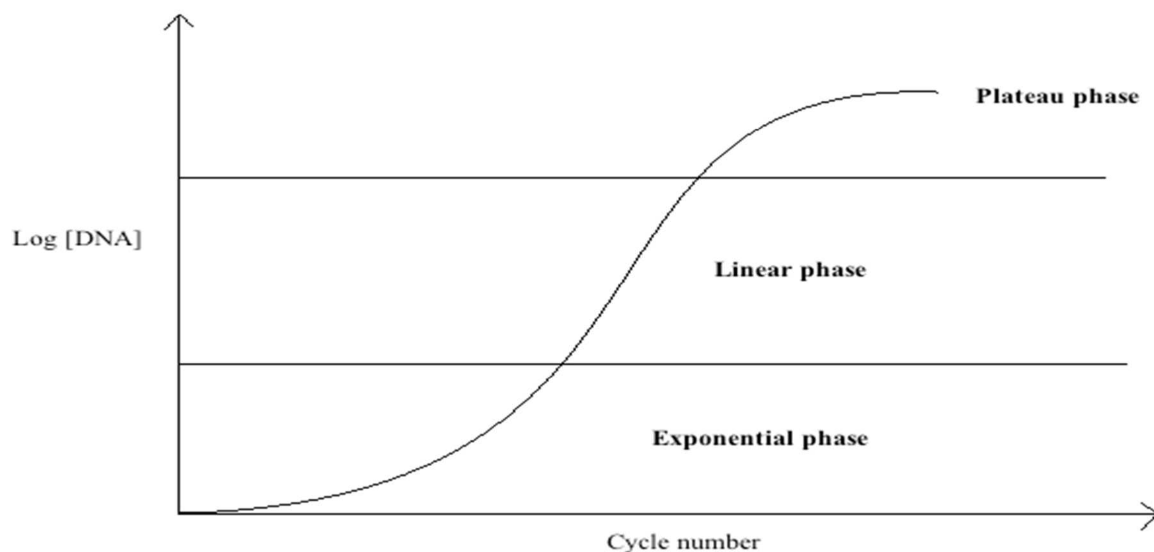


**Figure 3.** The three phases of PCR

### *2.4.3.4 Real-time PCR*

In normal PCR reactions, results are obtained from gel electrophoresis that is stained with a dye. Bands are formed with varying brightness according to the number of copies made. With real-time PCR, a detector records the reactions, which can be seen in real-time. The generation of fluorescent light increases as more copies of the DNA are produced during the cycles.

The advantages of real-time PCR are that the efficacy can be precisely calculated, the automation of the melt curve analysis and there is a true quantitative analysis of gene expression.

Compared to conventional PCR, real-time PCR is less time consuming, less cross-contamination occurs and automation is possible. In traditional serotyping methods it is possible to determine the subspecies of *Salmonella* but the serovar cannot be classified. In the study performed by Malorney et al., 2007, detecting of *S*. Enteritidis in pooled eggs, real-time PCR was employed using sefA target gene (encoding a fimbrial antigen). *S*. enterica ssp. enterica strains in group D also possess this gene which could mean false positive results of *S*. Enteritidis (Seo et al., 2004). Therefore in the experiment performed by Malorney et al., the Prot6e gene was set by the primer which is *S*. Enteritidis specific located on 60-kb virulence plasmid which encodes a particular surface fimbriae characteristic for *S*. Enteritidis. To control false negative results of the experiment, internal amplification control was used. A pre-enrichment stage for 18-20 hours, a DNA extraction step followed by the real-time PCR step took approximately 24 hours that can be compared to the traditional culture and serotyping methods that take at least 5-6 days. The identification of *Salmonella* Enteritidis in whole chicken carcasses by real-time PCR showed 100% accuracy of the 25 samples tested. Even though traditional serotyping methods are relatively accurate they are not able to identify specific serovars of *Salmonella*. For the identification of the serovars, molecular methods such as pulsed-field gel electrophoresis may be used. However, this is time-consuming and laborious and therefore not optimal in case of rapid results. Real-time PCR in the study performed by Malorny et al., 2007 showed high selectivity and maximum accuracy.

*Salmonella* serotype Typhimurium and Enteritidis are able to be isolated from both living chickens and in humans consuming infected meat. Gastroenteritis caused by non-typhoidal salmonellosis is a problem worldwide and therefore the detection of microbial infection is important for identifying a deficiency in the handling of chicken meat. Critical stages where microbial growth occurs include evisceration, cooling, packaging and transport stages according to Rasschaert et al., 2008.

Multiplex real-time PCR was used in the experiment performed by de Freitas et al., 2010, for the rapid detection of *Salmonella* species in chicken meat to reduce diagnosis time. By avoiding the conventional methods there is no need for viable number of cells for detection since PCR can generate enough DNA copies. It must however be noted that PCR cannot differentiate between viable and dead cells since it only uses the DNA as a template. The differences between conventional methods and PCR could therefore be due to injured cells that cannot be cultured but detected by PCR. Out of 200 samples (127 poultry carcasses and

73 poultry viscera) the mPCR (multiplex real-time polymerase chain reaction) was able to detect species of the *Salmonella* genus in 2,74% of the samples in the study and showed the same accuracy as in the traditional methods. However, the traditional methods of detection were not able to differentiate the serotype. In 1,37% of the samples *S*. Enteritidis was detected by mPCR. All positive control samples could be identified and the molecular and conventional methods yielded the same results for the positive and negative samples. The results showed that the PCR reactions were 98% specific for each serotype. Kumar et al. in 2006 obtained similar results from chicken meat broth as well as Lee et al. in 2009 where multiplex real-time PCR detected *S*. Typhimurium and Enteritidis serotypes in pork and beef. The detection of *Salmonella* in the carcass and viscera of chicken meat with conventional and multiplex real-time PCR methods yielded similar results but the experiment indicated that the quality of the diagnostic methods of PCR and mPCR was high and would yield industries and producers due to the rapid detection. This would enable them to take the measures needed quicker to avoid further contamination.

Real-time PCR was applied for the detection of *Salmonella* species in food products in the experiment performed by Su Hwa Lee et al., 2009, Out of 128 pork meat samples, 110 were inoculated with *Salmonella* isolates. The results showed that all 110 *Salmonella* species could be detected but not the 18 non-salmonella species. The specificity was 100%, 100% and 99,1% respectively for *Salmonella* spp., *S*. Typhimurium and *S*. Enteritidis and the sensitivity showed 100%, 100% and 91,7% respectively. Conclusively the study showed that the multiplex real-time PCR method was able to sensitively detect *Salmonella* spp. and differentiate between *S*. Typhimurium and *S*. Enteritidis in meats. The real-time PCR method was limited to detect a single strain of *Salmonella* species whereas the multiplex real-time PCR was able to "apply the assay to a diagnostic purpose compared to the real-time PCR assays" (Su Hwa Lee et al., 2009).

Akiba et al., 2011 performed a similar experiment using 118 serovars of *Salmonella* species and 12 non-Salmonella serovars using the *Salmonella* specific invA gene for confirmation. Serovar-specific genomic regions (SSGRs) were used which are highly prevalent among wild type strains of the target serovars. These were selected using comparative genomics approach Even though a small number of false positives were observed with mPCR, no false negatives could be observed.

In 2011, Zhang et al. compared a traditional method with three molecular methods of detecting *Salmonella* in vegetables. Different vegetables were inoculated with *Salmonella* serovars at different levels. The results revealed that the molecular methods were equally successful with the traditional method in detecting the *Salmonella* species. Their conclusion declared that quantitative real-time PCR has the potential to be used as initial screening step whereas quantitative reverse transcriptase real-time PCR has the potential to detect only viable *Salmonella* cells because after the enrichment step only positive samples are obtained (indicative of viable cells).

# 3. Materials and methods

## 3.1 Bacterial strains and samples

### *3.1.1 Bacterial strains*

The bacterial strains used in this study are *Salmonella* Enteritidis (NCAIM B.01908) and *Salmonella* Typhimurium (ATCC13311). The origin of the *Salmonella* serotypes (*S*. Cerro, *S*. Infantis, *S*. Newport, *S*. Tennessee, *S*. Abony) was obtained from the Institute of National Food Chain Safety Office, the Food and Feed Safety Directorate and Food Microbiology Reference Laboratory in Budapest, Hungary.

### *3.1.2 Samples*

The samples tested (raw broiler meat samples) were purchased from the local market in Budapest.

## 3.2. Enrichment broth

Rappaport-Vassiliadis enrichment broth was used for the enrichment and selective isolation of *Salmonella* species in this study. Rappaport et al. was the first to formulate the enrichment that was later modified by Vassiliadis et al by increasing the incubation temperature from 37 $^{o}$

C to 43°C. It was later found by Peterz that incubation at 41,5°C for 24 hours improved the obtainment of Salmonella.
The enrichment broth consists of soy peptone (carbon and nitrogen source for growth), magnesium chloride that raises the osmotic pressure in the medium and potassium phosphate that acts like a buffer. Malachite green in RV broth inhibits growth of other organisms apart from *Salmonella*.

The RVS (Rappaport-Vassiliadis Soy) broth is a modification of the Rappaport-Vassiliadis broth for increasing the reliability of the broth. In earlier studies, it has been shown that RVS broth yields more positive *Salmonella* samples when investigating chicken carcasses compared to other enrichment broths (Muller-Kauffman tetrathionate with novobiocin – MKTTn) indicating higher sensitivity and specificity (Hyeon et al., 2012)

### 3.3 Examination of broiler meat samples with redox potential method

To investigate the sensitivity of the examination technique 25 g of broiler meat sample was artificially inoculated with *Salmonella* Enteritidis and *Salmonella* Typhimurium and added to 225 ml RVS broth and the mixture was homogenized and incubated at 42°C. For the testing, the MicroTester was used which is a 32-channel redox potential measuring equipment using a water bath thermostat with ±0,2°C accuracy, test cells (250 ml measuring cells equipped by Schott Blue Line 31 RX redox-electrodes), PC drive (Windows XP using a special software), data collection/evaluating unit and a monitor.
In the measuring cell the redox potential is detected as the proliferation of microbes increases ($10^6$-$10^7$ cfu/ml) until a critical cell concentration (Nc) is reached. When the rate of change (dE/dt) in absolute value overcomes a definite detection criterion (DC) the time to detection (TTD) can be determined. There is a linear relationship between the time to detection and the logarithm of the initial viable cell count (log Ni). A calibration curve can be made which makes it possible to estimate the initial viable cell count as a function of the TTD (Erdősi et al. 2014).

## 3.4 Examination of broiler meat samples with Real-time PCR

### *3.4.1 DNA isolation*

Genomic DNA was extracted from 1 ml of enriched food sample that was positive according to the result of the redox potential measurement. For the isolation, *Mericon* DNA Bacteria kit (Qiagen) was used; the procedure was as following:

1. 1 ml of the incubated enrichment culture was pipetted into a 2 ml microcentifuge screw-cap tube and centrifuged at 13,000 x g for 5 minutes.
2. The supernatant was discarded using a pipet.
3. 200 µl Fast Lysis Buffer was added to the bacterial pellet and resuspended by vigorous vortexing.
4. The microcentifuge tube was put into a heating block set to 100 º C and was heated for 10 minutes.
5. The sample was removed and set to cool off for 2 minutes until reaching room temperature.
6. The tube was centrifuged at 13,000 x g for 5 minutes.
7. 100 µl of the supernatant was pipetted into a 1,5 ml microcentrifuge tube. A part of the collected supernatant was directly used in a PCR reaction.

### *3.4.2 Real-time PCR assay*

The Real-time PCR amplification was performed on SLAN® Real-Time PCR System (Hongshi) and was performed in 20 µl reaction volume.

2 µl DNS template
10 µl 2x thermo scientific Luminaris Color Probe High Rox qPCR Master mix
0,6 µl reverse primer
0,6 µl forward primer
0,4 µl Probe
Nuclease free water added to total 20 µl
The primers and probes were synthesized by the *Sigma-Aldrich* company.

1. 10 µl of the Luminaris Color Probe High Rox qPCR Master mix was mixed with 0,6 µl reverse primer, 0,6 µl forward primer, 0,4 µl Probe and 6,4 µl Nuclease free water in a tube and put in room temperature.
2. After mixing thoroughly the mixture was dispensed into PCR tubes.
3. 2 µl DNA template was added to the PCR tubes.
4. The reactions were mixed gently to not cause bubbling.
5. The samples were placed in the PCR cycler and the program was started.

A two-step protocol was used which included a UDG pre-treatment at 50°C for 2 minutes, an initial PCR activation step, activation of HotStarTaq *plus* DNA Polymerase for 10 minutes in 95°C, a 40 –step denaturation and annealing/extension for 60 seconds at 60 °C. Fluorescence detection was performed at the end of the annealing stage of each cycle.

**Table 2.** *Salmonella* spp. serotypes, the target genes (the fliC and sefA genes were used as primers in the following sequences), the name of the genes and the sequences to be amplified.

| Species | Gene | Name | Sequence (5′ - 3′) |
|---|---|---|---|
| *Salmonella* spp. | 16s rRNA | S16R-F | aggccttcgggttgtaaagt |
| | | S16R-R | gttagccggtgcttcttctg |
| | | Scom-FAM | [6FAM]-aaccgcagcaattgacgttaccc-[BHQ1] |
| *Salmonella* Typhimurium | *fliC* | SfC-F | tgcagaaaattgatgctgct |
| | | SfC-R | ttgcccaggttggtaatagc |
| | | ST-JOE | [JOE]acctgggtgcggtacagaaccgt[BHQ2] |
| *Salmonella* Enteritidis | *sefA* | SsA-F | ggtaaaggggcttcggtatc |
| | | SsA-R | tattggctccctgaatacgc |
| | | SE-Cy5 | [Cy5]-tggtggtgtagccactgtcccgt-[BHQ2] |

The fliC flagellin gene encodes specific components in the flagellum of *S*. Typhimurium and the sefA gene specifically encodes *Salmonella* Enteritidis fimbrial protein.

The amplified target sequences were detected using fluorescent probes specific for each target. When the amplified products are detected there is an increase in the fluorescent signal from the bound probes. The detection in real-time was possible by monitoring the increase in fluorescence during the PCR cycles.

# 4. Results and discussion

## 4.1.Redox potential measurement

The time for detecting one target microorganism (N=1) in the measuring cell can be determined from the intercept of the calibration curve (logN=0). It can be assumed that the cell is free of microorganisms if no TTD can be reached. Previous studies have demonstrated that the calibration curves for several *Salmonella* serotypes show different detection times where *S*. Newport in RVS broth had the longest detection time (23,5 hours in broth without added food).

The calibration curve of *Salmonella* Newport is shown in Figure 4.

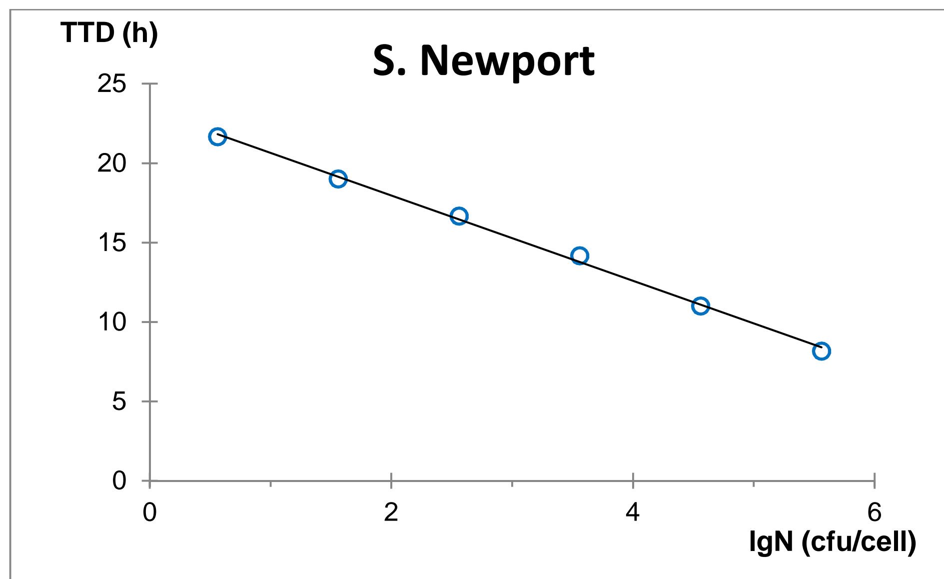


**Figure 4**. Calibration curve of *Salmonella* Newport.

Equation of calibration curve of *Salmonella* Newport:

TTD= -2.6857·logN + 23.332

$R^2 = 0.9976$

The measuring cell can be considered pathogen-free if the detection time cannot be determined, based on the slowest proliferating serotype of *Salmonella*. The examination duration can therefore be determined according to the detection of a single cell of the slowest growing serotype (Erdösi et al, 2014).

The calibration curves of *Salmonella* Enteritidis and Typhimurium are shown in figure 5.

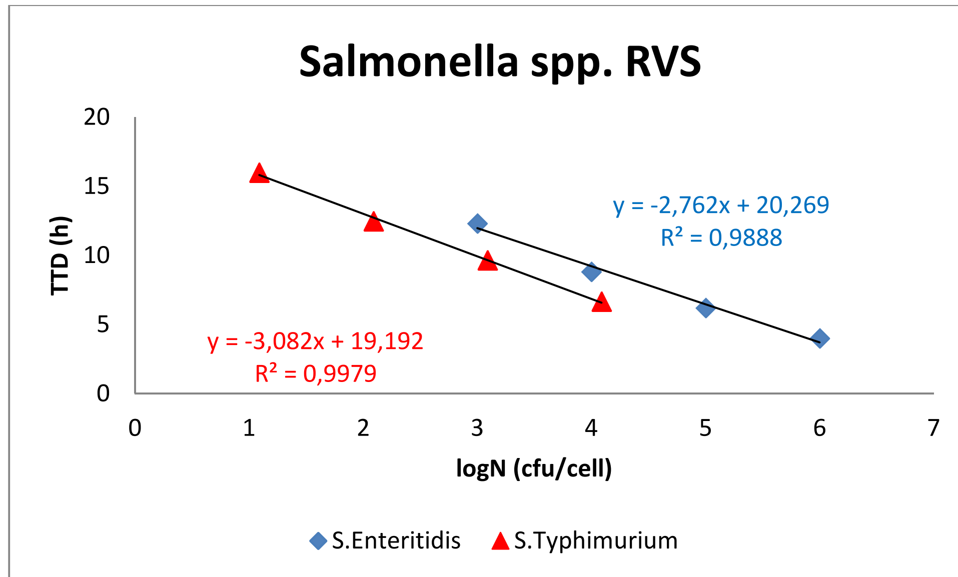


**Figure 5.** Calibration curves of *S*. Typhimurium and *S*. Enteritidis obtained from redox potential measurement (T=42 °C)

The equation for *S*. Typhimurium:

$TTD=-3.082 \cdot logN+19.192$

$R^2 = 0.99795$

The equation for *S*. Enteritidis:

$TTD=-2.762 \cdot logN+20.269$

$R^2 = 0.9888$

The proliferation of *S*. Typhimurium and *S*. Enteritidis were examined in broiler meat samples in RVS broth.

In the redox potential measuring, the time to detection (TTD) varied for the different samples. The time requirement of initial single cell detection by the redox potential measurement in 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 until detection. Only the positive samples need the PCR identification The results (positive or negative) and time requirement of the redox potential measurements and real-time PCR are summarized in Table 3.

**Table 3.** Detection of *S.* Typhimurium and Enteritidis in fresh broiler meat (mean of 3 parallels)

| | TTD (h) | Real-time PCR | | | Detection time (h) |
|---|---|---|---|---|---|
| | | *Salmonella* spp | *S.* Typhimurium | *S.* Enteritidis | |
| Chicken meat | 7,8 | + | - | - | **11** |
| Chicken meat | 15,5 | + | - | - | **18,5** |
| Chicken meat + *S.* Enteritidis | 3,3 | + | - | + | **6,5** |
| Chicken meat + *S.* Typhimurium | 10,0 | + | + | - | **13** |
| Chicken meat + *S.* Newport | 3,1 | + | - | - | **6,5** |
| Chicken meat + *S.* Infantis | 3,3 | + | - | - | **6,5** |
| Chicken meat + *S.* Newport, *S.* Infantis, *S.* Cerro, *S.* Tenessee, *S.* Abony | 3,5 | + | - | - | **6,5** |
| Chicken meat + *S.* Newport, *S.* Infantis, S. Cerro, *S.* Tenessee, *S.* Abony, *S.* Enteritidis | 3,17 | + | - | + | **6,5** |
| Chicken meat + *S.* Newport, S. Infantis, *S.* Cerro, S. Tenessee, S. Abony, *S.* Typhimurium | 3,17 | + | + | - | **6,5** |
| Chicken meat + *S.* Newport, *S.* Infantis, *S.* Cerro, *S.* Tenessee, *S.* Abony, *S.* Enteritidis, *S.* Typhimurium | 3,17 | + | + | + | **6,5** |
| Sterile RVS | - | | | | **24** |

According to several earlier studies, real-time PCR has shown efficient for the determinative serotyping of *Salmonella* species. The results have shown high accuracy and selectivity and the use of multiplex real-time PCR has become favoured.

The analytical reference method for this criterion is the EN/ISO 6579 (for detection) and White- Kaufmann-Le Minor scheme (for serotyping). The time requirement of the conventional method and the combined method is shown in Table 4.

**Table 4.** Time requirement of the reference method and the combination of the redox potential measurement and real-time PCR method

| | Time requirement (h) | |
|---|---|---|
| | positive samples | negative samples |
| EN/ISO 6579 + White- Kaufmann-Le Minor scheme | 162-282 | 66 |
| redox + real-time PCR | 27 | 24 |

## 6. Conclusion

In this study the possibility of rapid and reliable detection of *S.* Enteritidis and *S.* Typhimurium was demonstrated by combination of the redox potential measurement and real-time PCR methods. In order to detect low number of bacteria and resuscitate physiologically stressed and injured ones, the enrichment phase was a crucial step. During the enrichment phase the *Salmonella* positive samples could be screened by the redox potential measurement technique. Instead of biochemical and serological confirmation, further identification was carried out by applying real-time PCR technique.

In case of negative samples, the results could be obtained in 24 h by the redox potential measurement. Only the positive samples need the expensive PCR identification. The primers used were effective in the amplification of the fliC and sefA genes for *Salmonella* serotypes Typhimurium and Enteritidis respectively.

It is conclusive that it would be beneficial for food manufacturers to use this method for detection of *S*. Enteritidis and Typhimurium in fresh poultry meat. A comparison can be made of the detection times; maximum 24 hours for the combination of the redox potential measurement and real-time PCR methods whereas 162-182 hours is needed for detection in conventional methods which confirms that the method presented in the study would aid the

producers by effectively determining the absence of *Salmonella* Enteritidis and Typhimurium in 25 g sample of fresh poultry meat. The high number of *Salmonella* outbreaks across the world is in need of fast microbiological methods and the combination of the redox-potential method and the real-time PCR presents rapid and reliable results that can be useful for testing products at a low cost and time to detection.

In the experiment performed by de Freitas et al., 2010, the molecular and conventional methods were compared using a positive control and 200 samples of meat and viscera. The results showed that the two different methods had the same accuracy in detecting *Salmonella* species in the samples although the traditional method was not able to specify the serovar compared to the molecular. Most studies performed indicate that molecular methods are equivalent to traditional methods regarding accuracy of results.

The drawback of the quantitative real-time PCR is the detection of microbial DNA that can be found after the target cells are dead whereas the traditional methods only detects viable cells present.

The combination of the redox potential and PCR methods is labour-saving and significantly decreases the cost and time of detection of *Salmonella* Typhimurium and Enteritidis.

# 7. Summary

Salmonellosis is one of the major foodborne diseases causing hundreds of thousands of cases each year in the EU and therefore rapid detection methods are important for determination in foodstuffs. The EU has implemented an integrated approach to reduce its occurrence. Since then, Salmonellosis has decreased by 50%.

*Salmonella* (S.) Typhimurium and *S*. Enteritidis are two causative agents responsible for some of the major foodborne diseases occurring worldwide. The necessity of fast and reliable detection methods of low cost has become of utmost importance in order to rapidly identify incidences of *Salmonella* contaminations of foodstuffs. *Salmonella* Enteritidis and Typhimurium must be absent in 25 g fresh poultry meat sample placed on the market during their shelf-life; out of 5 samples 0 may be positive, according to the 2073/2005 legislation to ensure public health.

The detection in foods is possible by conventional (traditional) and rapid microbiological methods. Conventional methods include pre-enrichment, selective enrichment, selective agar media and biochemical and serological tests. Although accurate, this is laborious and takes several days to perform and obtain results (162-282 h)

Molecular methods have shown to be rapid and produce accurate results and could be the future for fast investigations of contaminated foodstuffs.

In this study the combination of redox potential measurement based method and real-time PCR method was used for the detection of *Salmonella* Enteritidis and Typhimurium. During the enrichment phase the *Salmonella* positive samples could be screened by the redox potential measurement technique. Instead of biochemical and serological confirmation, applying real-time PCR technique carried out the further identification. The combination of redox potential and real-time PCR can accomplish results in maximum 27 hours compared to conventional methods that can take up to 162-182 hours which indicates that the combined method may be favourable for the effective and rapid identification of *Salmonella* Enteritidis and Typhimurium in poultry meat samples.

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