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Introduction of a real-time PCR instrument in the Debrecen laboratory of the National Food Chain Safety Office for Salmonella detection

Keywords: *Salmonella*, salmonellosis, real-time PCR, detection of pathogenic bacteria

SUMMARY

Salmonellosis is one of the most widespread zoonoses, being the animal disease spreading to humans encountered most often all over Europe [1]. Consequently, the detection of Salmonella spp. in foods can be classified as a microbiological analysis of primary importance in the Debrecen Regional Food Chain Laboratory of the National Food Chain Safety Office, using the horizontal method. However, the Salmonella detection method according to standard MSZ EN ISO 6579-1:2017 requires a long time, at least 3 working days. Therefore, it was decided in the Debrecen Regional Food Chain Laboratory of the National Food Chain Safety Office to introduce a rapid method based on the use of a real-time PCR instrument. The applicability of the method was tested on the basis of the results of Salmonella detection in meat samples. There was little difference when comparing the results obtained using horizontal and molecular biological methods. The results of the two methods were 99% identical. Considering the efficiency of real-time PCR (99.3%), encouraging conclusions can be drawn regarding the introduction of the new method.

Introduction

With the rise in living standards, the number of food safety requirements increases and they become more stringent. In relation with this, the detection of pathogens of food industrial source in the food chain process is important, for which fast and reliable methods must be used **[2, 3]**. Sufficient speed, acceptable precision and sensitivity of the methods used are essential both for safe food supply and the timely informing of consumers in case of a recognized emergency **[3, 4]**.

Literature review

Characteristics of the genus Salmonella

The genus *Salmonella* consists of facultative anaerobic, Gram-negative, linear, rod-shaped microorganisms of the *Enterobacteriaceae* family moving with peritrichous flagella, with more than 2,500 serotypes known **[5, 6]**. They are possible inhabitants of the human and animal alimentary canal, and when released into the environment, they can be found in waters and soil, on plants and in

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raw materials of animal origin. *Salmonella* species proliferate at temperatures between 6 and 47 °C and in the pH range 3.8-9.5, and can survive long periods under dry conditions, thus remaining viable for months or even years for example in egg powder or dry pasta **[7]**. In dry straw on which poultry litter is based, *Salmonella* can remain viable for up to 11 months **[8]**. For this reason, it is important to comply with the general and hygiene rules applicable to animal farming.

Salmonellosis

Since the 1990s, considerable attention has been paid to *Salmonella* srtains, which play an important role in the development of infectious diseases **[9]**. Overall, it can be said that of the 2,500 *Salmonella* serotypes, the ones that merit attention from a public health point of view are those that cause paratyphoid in animals and diseases of varying course and severity in the human body. These include *Salmonella* Enteritidis and *Salmonella* Typhimurium, the microorganisms that cause the most prevalent human disease in the world **[1, 10]**.

Most often the pathogen enters the environment with the faeces of animals or humans that are either diseased or asymptomatic, but excreting the pathogen. The symptoms of salmonellosis appear within 6 to 48 hours after the consumption of food contaminated with *Salmonella*, and they include in mild cases headache, lethargy, vomiting and diarrhea. In individuals with a strong immune system, these symptoms disappear within 2 to 5 days without medication, but in some cases, latency and recovery may take a longer time. In severe cases, the consumption of food contaminated with *Salmonella* can cause a high fever and even septicemia **[11, 12]**. More than half of human salmonellosis cases are infections caused by poultry, especially by eggs contaminated with faeces. Other sources of infection can be any raw material of animal origin, drinking water, fruits and vegetables that are either contaminated with faeces or are unwashed, seasonings and spices **[1]**. An asymptomatic form of the disease is also common, during which the pathogenic *Salmonella* bacterium proliferates in the alimentary canal and then excreted from the body with the faeces. In the event of a breach of personal and manufacturing hygiene, food consumers may be infected by faecal *Salmonella* via the oral route through foods or their raw materials (faecal-oral transmission) **[13]**. Further risks include the distribution and consumption of contaminated and insufficiently heat-treated meat products, as well as the use and consumption of raw milk without pasteurization and without further heat treatment. In addition to improper heat treatment, the survival and proliferation of the pathogen may also be helped by the improper storage of foodstuffs in warm, humid places **[1, 11]**. An additional hygiene hazard is that, similarly to *Listeria monocytogenes*, *Salmonella* is also able to form a biofilm on metal, glass, rubber and plastic surfaces used in the food industry, therefore, continuous cleaning and disinfection of all equipment and the connecting piping, valves and other fixtures intended to come into contact with food is essential **[14]**.

Salmonellosis is not limited to being a human disease. The disease can also occur in animals, such as pigs or poultry. Related to this, a *Salmonella* eradication program has been in place in poultry flocks in Hungary since 1997 against *Salmonella* Enteritidis and *Salmonella* Typhimurium **[11]**. In addition to the correct choice of livestock farming technology, continuous health monitoring and compliance with hygiene rules, an integrated protection program was also required. Using Council Directive 92/117/EEC and World Health Organization guidlines, the so-called "White book" was prepared, a publication titled „An integrated quality management system for the control of *Salmonella* in the poultry sector in Hungary" **[15]**.

An important part of *Salmonella* eradication is to prvide animals with drinking water and feed that is *Salmonella* free. Sources of infection among feed raw materials may include oilseeds, during the processing of which conditions favorable for the permanent colony formation and proliferation of *Salmonella* could develop through the humid, dusty and warm climate of the extraction plants **[16]**.

The main supervisor of *Salmonella* monitoring in Hungary is the National Food Chain Safety Office (NÉBIH), which performs full *Salmonella* testing of livestock, meat and various foodstuffs with its official laboratories and with the involvement of certain officially approved provate laboratories **[11]**.

Real-time PCR

The availability of reliable, fast and accurate detection methods has become increasingly important for agriculture and the food industry in recent years. Such methods allow the simultaneous detection of several pathogenic microorganisms and provide validated results, similar to the "conventional" microbiological methods described in the standards **[17]**.

The polymerase chain reaction (PCR) is the basis for a rapid diagnostic method that is used to detect the pathogenic DNAthat is suspected to be present in the sample, and so it can also be used for the direct detection of *Salmonella* bacteria in food samples **[18]**. There are several variatons of the PCR method, including the real-time PCR technique used by us. Compared to the conventional polymerase chain reaction, in the case of the real-time technique, amplification and detection are accomplished simultaneously thanks to the fluorescent moieties used. During the measurement, a fluorescent signal is emitted by the highlighted DNA sections of the sample, which can be detected by the system,

thus making the detection process faster and more sensitive than the „conventional" methods **[19, 20]**. By today, real-time PCR technology has become a routine laboratory analysis. The net time requirement for testing a sample package consisting of 50 items can be reduced to 9 to 10 hours **[21]**.

Materials and methods

Measurements were carried out in the Debrecen Regional Food Chain Laboratory of the National Food chain Safety Office (Food Chain Safety Center Nonprofit Kft.) between January and May 2018.

Kit and equipment used for real-time PCR

- Bio-Rad iQ-Check *Salmonella* II kit: reagents are sufficient for 96 tests
  - Reagent A: lysis reagent 1×20 ml
  - Reagent B: fluorescent probe 1×0.55 ml
  - Reagent C: amplification mix 1×4.4 ml
  - Reagent D: negative PCR control 1×0.5 ml
  - Reagent E: positive PCR control 1×0.25 ml
- bioSan TS-100 Thermo-shaker
- Sigma 1-16 centrifuge
- Velp scientifica (Rx^3) vortex mixer
- Yellowline MST basic C magnetic stirrer
- Bio-Rad CFX93 Real-Time System PCR instrument
  - C1000 Touch Thermal Cycler
- pipet4U pro automatic pipette

Method

Sample preparation

During sample preparation, in the case of ground or prepared (marinated, seasoned) pork or beef, 10 g of the sample was weighed on a laboratory balance, while 25 g of the sample was weighed in all other cases.

Following a 10:1 ratio, 25 g samples were weer filled to 250 g and 10 g sample were filled to 100 g with buffered peptone water, then it they were suspended in a Stomacher machine for 30 seconds.

Detection of Salmonella spp. using the real-time PCR method

Detection was performed according to the description of the MB/12/2010 *Salmonella* kit. Bio-Rad iQ-Check *Salmonella* II kit was used for isolation. The kit contains 4 reagents, as described at the beginning of the ***Materials and methods*** section.

Following 18 ± 2 hours of incubation, DNA was isolated from a pre-enrichment fluid of buffered peptone water incubated at 37 °C. For some products, to prevent inhibition, it is advisable to add 20 µl of the pre-enrichment medium to 1 ml of the peptone water buffered at 37 °C, which should then be incubated at 37 °C for 4 ± 1 hours.

DNA extraction

DNA was extracted from the digested sample in a thermomixer at 99 °C. 1 ml of the incubated buffered peptone water was transferred into an Eppendorf tube, and it was centrfuged at 12,000 g for 5 minutes. During centrifugation, Reagent A was stirred o a magnetic stirrer.

Following centrifugation, the supernatant of the sample was removed, and 200 µl of Reagent A was added to the pellet, while the reagent was constantly agitated on the stirrer for homogenization. This was necessary, because the reagent use has a beaded structure and a uniform distribution needs to be ensured when used. The pellet and the Reagent A added were suspended with the help of a pipette, then it was vortexed for at least 20 seconds until the pellet was completely dissolved. The samples thus prepared were heat treated in a thermomixer for 15 minutes at 99 °C and 1,400 rpm. The samples were then taken out from the thermomixer and vortexed for at least 20 seconds while holding the top of the Eppendorf tubes, then they were centrifuged again at 12,000 g for 5 minutes. Finally, 50 µl of the supernatant was pipetted into an Eppendorf tube. Samples can be stored until the measurement at -20 °C for 1 day.

Real-time PCR

The reagents of the PCR plate were weighed in a sterile box. Reagents B and C were weighed into the sterile Eppendorf tube according to the table provided with the kit, including the positive and negative control samples, which are essential for the measurement. The reaction mixture put together can be stored at 4 °C for up to 1 hour.

45 µl amounts of the mixtures were added to the PCR plate wells at the following ratios:

- 5 µl/sample of Reagent B,
- 40 µl/sample of Reagent C.

Subsequently, 5 µl each of Reagent D (negative control), Reagent E (positive control) and the unknown sample were added, in an identifiable order, to the mixtures under the liquid level using a pipette. As a final step of the isolation, the plate was sealed and bubbles were removed.

Following this, the plate containing the reaction mixtures was placed in the Bio-Rad CFX93 Real-Time System PCR instrument. The polymerase chain reaction was run and the results were evaluated using the Bio-Rad CFX Manager Industrial Diagnostic Edition 2.2 program. For the detection, i.e., to achieve measurable signal size, 49 PCR cycles were required.

The device used by us was equipped with a FAM (Fluorescein amidite) filter. During the evaluation, the result is positive, if a characteristic sigmoidal curve crossing the threshold value is displayed. In most cases, if the value is positive, the curve will appear after the 30th cycle. For curves appearing during the 28th cycle (too early) or after the 40th cycle (too late), the reliability of the results obtained was not satisfactory. The affected questionable samples were also analyzed by the microbiological method described in teh standard.

Calculation of the efficiency of the PCR reaction

To calculate the efficiency of the polymerase chain reaction, the LinRegPCR: Analysis of quantitative RT-PCR Data software was used, which calculates the individual reaction efficiency for a particular gne tested and averages the results to give the total reaction efficiency. Its advantage is that it does not require a separate standard curve, since it uses the slope of the straight line fitted to the linear portion of the amplification curve plotted in a logarithmic view in the following formula:

$$E = 10^{(-\frac{1}{m})} - 1 \times 100,$$

where „E" is the efficiency and „m" is the slope **[22]**.

In order to verify the efficiency, relative accuracy, specificity and sensitivity were also calculated for all of the samples tested using the formulas contained in standard MSZ EN ISO 1640:

- Relative accuracy (%): $\frac{(p+n)}{N} * 100$
- Relative specificity (%): $\frac{n}{(n+fp)} * 100$
- Relative sensitivity (%): $\frac{n}{(n+fp)} * 100,$

where „N" is the total number of samples tested, „n" is the number of negative samples, „p" is the number of positive samples, „fn" is the number of false negative samples and „fp" is the number of false positive samples **[23]**.

Results and evaluation

Sample preparation, DNA isolation

In the course of our experiments, a total of 109 samples were tested for *Salmonella* using a real-time PCR instrument. Grouping of the samples is shown in **Table 1**. The majority of the samples to be examined were poultry, namely chicken and turkey neck skin, meat and meat products.

1 ml of the incubated buffered peptone water was used for DNA isolation, the rest was used when applying the horizontal method. The incubated buffered peptone water samples were stored in Eppendorf tubes in a refrigerator at 4 °C until use. The optimal proliferation temperature for *Salmonella* is 37 °C, but it can proliferate between 5 and 47 °C. Our subsequent experince has shown that storage at low temperatures may have inhibited proliferation, but a significant proportion of bacterial cells have remained viable.

Results of the detection

Samples were evaluated using the Bio-Rad CFX Manager Industrial Diagnostic Edition 2.2 program. All results are summarized by the program in a report.

For the assay using the real-time PCR method, all sample preparation steps were carried out according to the protocol provided with the kit. During the observation, a total of 8 measurements were performed. In the first series of measurements, the expected results of the samples were known, since the detection had already been carried out previously using the horizontal method. Following this, with the exception of one measurment, the detection results of *Salmonella* obtained using real-time PCR, i.e., the molecular biological method, were identical to the results obtained with the horizontal method. The first and only difference between the two methods was found in the case of the fourth measurement. As a result of the bacterial detection by the molecular biological method, the fresh, ground turkey drumstick fillet was positive, whereas the classical method gave a negative result. When using the real-time PCR technique, inconclusive samples and those that are positive for *Salmonella* require a „classical" confirmation test, which was performed according to the method prescribed by standard MSZ EN ISO 6579-1:2017. The experiment of Wang-Mustapha was aimed at the elimintation of false positive results, during which *Salmonella* detection was carried out on hen eggs. As an addition, during the sample preparation and isolation of the conventional PCR technique, ethidium bromide monoazide was used to stain dead bacterial cells. In the stained bacterial cells, the reaction between the DNA polymerase enzyme and the hereditary material is prevented by the ethidium bromide monoazide, thus, their DNA content is unable to participate in the polymerase

chain reaction. This way the probability of false positive results caused by dead *Salmonella* cells can be effectively reduced **[24]**.

Most of the samples received by the laboratroy were poultry meat. Among these, both in the case of chicken and turkey samples, neck skin analyses were carried out in the largest numbers. The need for testing neck skin is justified, since in 2011, 86% of broiler chicken neck skin samples were positive for the presence of *Salmonella* **[25]**. As shown in ***Figure 1***, the pathogenic bacterium could be detected in 84.6% of the chicken samples, including neck skin, wings and fresh whole chicken leg, examined by us using the real-time PCR instrument. Of the 44 samples of turkey meat and neck skin, 10 postive results were obtained, including positive results from neck skin, ground drumstick fillet and breast. Like guineafowl, turkey breast came to the laboratory for testing frozen. The presence of *Salmonella* could be detected in both meats. This confirms the fact that although *Salmonella* is unable to proliferate below 5 °C, it remains viable, as inactive cells were activated during the pre-enrichment before the test and the pathogenic microbe began to proliferate.

Of poultry, duck, goose and pheasant meat were also received for *Salmonella* detection. All of these samples were found to be negative.

In the case of red meat, mainly pork was examined, as well as beef and game meat. Fish samples arrived for examination mostly forzen, but their number was negligible. A total of 29 samples belonged to the red meat and fish category, all of which provided negative results by both the horizontal and the molecular biological method.

The distribution of the postive and negative test results by meat type is shown in ***Figure 1***. It should be noted that a comparison between the meat and meat product samples was not possible because of the hectic distribution of the samples received by the laboratory.

Based on the comparison of the results obtained by us using the real-time PCR technique and the results obtained in the course of *Salmonella* detection according to standard MSZ EN ISO 6579-1:2017 it can be stated that the introduction of the use of a real-time polymerase chain reaction instrument at the Debrecen Regional Food Chain Laboratory is beneficial. A simple sample preparation protocol is provided by the manufacturer with the BioRad iQ-Check *Salmonella* II kit used, thus the assay process can be mastered easily, but DNA isolation and plate assembly require disciplined, accurate work.

The PCR technique allows the detection in mumltiple samples in less time than the „conventional" method. While the horizontal method takes 3 days in case of a negative result and 5 days in case of a positive result, the procedure based on the use of the real-time PCR instrument requires approximately 3.5 hours for the analysis of 20 samples, asuming negative results. Testing may take longer if the result is inconclusive using the molecular biological method, or if the sample is positive for *Salmonella*. In these cases, the detection must be repeated using the horizontal method. With real-time PCR, one can analyze up to 40 samples in one working day: while the first sample series is analyzed by the instrument, DNA isolation and plate assembly for the next sample series can be carried out.

Efficiency of the PCR reaction

In connection with the introduction of the new method, a total of 109 samples were tested for *Salmonella*, the result of which difered only in a single case from the result obtained using the horizontal detection. The false positive results suggest that the real-time PCR method is much more sensitive than the classical method. However, cross-contamination or the presence of dead *Salmonella* cells can lead to false positive results.

The effectiveness of the reaction was not only evaluated by observing and comparing the results. For this purpose, the LinRegPCR: Analysis of quantitative RT-PCR Data software was used, with the help of which the reactio efficiency was obtained as a percentage. During the evaluation, it was found that the efficiency of the polymerase chain reaction was either too low (below 88%) or too high (above 105%) for low sample number measurements. One of the reasons for this is the pipetting error or the fact that the mastermix has more primer than the optimum amount, considering the sample number to be examined.

Results read by the evaluation software are illustrated in ***Figure 2***. It can be clearly seen that the positive samples and positive control samples give a logarithmic curve and the efficiency is calculated by the program from the slope of the straight line fitted to the linear section of these curves, using the following formula:

$$E = 10^{\left(-\frac{1}{m}\right)} - 1 \times 100,$$

where „E" is the efficiency and „m" is the slope **[22]**.

Efficiency analysis was also performed by measurement, and total reaction efficiency was also calculated. The same or very close ratios were found for both measurement methods, and by evaluating all of the results, a final result of 99.3% efficiency was obtained.

To check the efficiency, relative accuracy, relative specificity and relative sensitivity were also calculated, the results of which by sample are shown in ***Table 2***.

Numerous studies have been published on the detection of *Salmonella* usubg the polymerase chain reaction, which also analyze the effectiveness of the method. The authors of these articles worked with a known *Salmonella* titer (CFU/25 g sample) during their studies, so they were able to perform more accurate efficiency calculations compared to our experiments **[26, 27, 28].**

Conclusions

The objective of our research was to substantiate the necessity and advantage of the real-time PCR instrument to be introduced at the Debrecen Regional Food Chain Laboratory of the National Food Chain Safety Office (Food Chain Safety Center Nonprofit Kft.). In the course of the experiments, detections were carried out in meat, neck skin and meat products, since salmonellosis is primarily caused by these foodstuffs. In the laboratory, *Salmonella* detection according to standard MSZ EN ISO 6579-1:2017 is one of the main microbiological tests to date, as it is one of the most well-known pathogenic bacteria that can cause mild or even severe symptoms when entering the body.

The detection of the bacterium was performed in parallel using the horizontal method and by a real-time PCR instrument in the same samples, and then the results obtained using the two methods were compared. In conclusion, it can be stated that a different result compared to the result of the detection performed using the method according to the above-mentioned standard was obtained in a single case out of 109. According to our results, real-time polymerase chain reaction is a reliable and accurate method. In addition, the advantages of the introduction and use of the instrument include simplicity, ease of use, low space requirement, as well as less chemical and material consumption. A further advantage is that the method is fast, as test results for 20 samples can be obtained within 3.5 hours. It is much more sensitive and accurate than the horizontal method, but it has the disadvantage that the presence of dead bacterial cells or high levels of sugar or fat in the foods may lead to false positive results.

To suport the advantagees of the molecular biological method, both individual and total reaction efficiency calculations were performed, with 99.3% being the result of the latter. In addition to efficiency, relative accurace, specificity and sensitivity were also determined, the results of which were 100%, 98.7% and 100%, respectively. Based on these, the efficiency of the real-time PCR method is considered to be excellent,

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