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RESEARCH ARTICLE

Comparison of Different Methods for the Detection of *Salmonella* spp. in Minced Meat Samples [1] [2] [3]

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Summary

The presence of *Salmonella* spp. in minced meat that is consumed in nine different sites of Istanbul is evaluated by using conventional culture (ISO 6579:2002), immunomagnetic separation (IMS) and fluorescent in situ hybridization (FISH) methods. *Salmonella* spp. was isolated from five of 50 (20%) minced meat with ISO 6579 method, and three of 50 (6%) minced meat using IMS method. Bacteria isolated from both ISO and IMS methods were identified as *Salmonella choleraesuis ssp. arizonae* and *Salmonella* spp. The presence of *Salmonella* spp. was determined from 37 of 50 (74%) minced meat by using FISH method. In the current study, it has been shown that ISO 6579 method was found more to be susceptible than IMS method for determining presence of *Salmonella* spp., FISH method is the best method to determine the presence of *Salmonella* spp. Even if the quick determination of the epidemics of international importance occured as a result of the contamination by pathogens derivated from foods, the results of the use of new methods should be supported by the conventional culture method.

Keywords: *Conventional culture method (ISO 6579 reference method), Fluorescent in situ hybridization (FISH) method, Immunomagnetic separation method, Minced meat, Salmonella*

Kıyma Örneklerinde *Salmonella* spp. Tespitinde Farklı Yöntemlerin Karşılaştırılması

Özet

İstanbul'un dokuz farklı semtinde tüketime sunulan kıyma örneklerinde geleneksel kültür yöntemi (ISO), immünomanyetik ayırma (İMA) ve floresanlı yerinde hibritleme (FISH) yöntemleri kullanılarak *Salmonella* cinsi bakterilerin varlığı tespit edilmiştir. İncelenen 50 kıyma örneğinin, geleneksel kültür yöntemi ile 5' inden (20%), İMA yöntemi ile 3' ünden (6%) *Salmonella* cinsi bakteri izole edilmiştir. Gerek geleneksel kültür yöntemi gerekse de İMA yönteminde besiyeri üzerinde üreyen kolonilerden elde edilen izolatlar *Salmonella choleraesuis ssp. arizonae* ve *Salmonella* spp. olarak tanımlanmıştır. İncelenen 50 kıyma örneğinin 37'sinde (74%) ise FISH yöntemi ile *Salmonella* cinsi bakterilerin varlığı tespit edilmiştir. Çalışmamızda *Salmonella* cinsi bakterilerin varlığını belirlemede geleneksel kültür yönteminin, İMA yöntemine göre daha duyarlı bir yöntem olduğu, FISH yönteminin ise *Salmonella* cinsi bakterilerin varlığını belirlemede en iyi yöntem olduğu tespit edilmiştir. Gıda kaynaklı patojenlerle kontaminasyon sonucunda ortaya çıkan uluslararası önemdeki salgınların hızlı tespiti ne kadar önemli olsa da, uygulanan yeni yöntemlerin sonuçlarının geleneksel kültür yöntemi ile desteklenmesi gerekmektedir.

Anahtar sözcükler: *Geleneksel kültür metodu (ISO 6579 referans yöntemi), Floresanlı yerinde hibritleme yöntemi (FISH), İmmünomanyetik ayırma yöntemi, Kıyma, Salmonella*

INTRODUCTION

Foods contaminated with microorganisms like pathogenic bacteria, parasitic helmints and protozoons cause great problems concerning the public health [1]. Another food type which bears great risk regarding the

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public health is meat and meat products. Under normal conditions, meat is sterile; however, it is contaminated according to cutting methods, cleanness of the knife used in the cutting process, water used in the cleaning process and to the preserving conditions and becomes dangerous regarding the microbial growth [2,3].

Salmonella bacteria are generally spread with food, contaminated with feces and are the primary factor for the food infection known as salmonellosis [4]. It is determined that in developed and developing countries, the main portion of salmonellosis cases, which are rated first in food infection and intoxication cases, involves consumption of contaminated animal originated food. For this reason, all stages of food production and consumption must be monitored and the food must be safe. Especially, in developed countries, the presence of *Salmonella* bacteria and serotype distribution in animal products such as minced meat containing the risk group are regularly monitored. Thus, a healthy database about proliferation of bacteria has been produced, and it has shed light on the epidemiological studies [5].

Generally, in order to determine the presence of *Salmonella* bacteria in foods, conventional culture method is used. With this method, results are obtained in nearly one week [6]. In food microbiology, new methods are needed to determine the specific bacteria and to confirm the lifeness of bacteria and known metabolic activities. Conventional culture methods which are used to examine water and food are deprived of the sensibility which is needed in direct determination of food pathogens. For this reason, before verification tests are applied, enrichment cultures are used to increase the number of pathogens which are in low number and identification of bacteria is performed after enrichment process [7].

Immunomagnetic separation (IMS) method is generally used in great efficiency in acquisition of damaged cells after enrichment process or in isolation of specific pathogen bacteria from heterogeneous cell suspensions [7-11]. Although IMS method takes the place of enrichment media, when it is used in combination with pre-enrichment and enrichment media, positive results are increased [12-14]. The first applications of IMS method in food microbiology are known to be performed in Brie (a salty and soft cheese type produced in Northern France), milk powder, yoghurt, meat, and vegetables [10].

For food microbiologists, direct monitoring of microbial populations in food products is an important issue. This is especially important when compared with trad conventional microbiological methods, which give results after a long time. In food samples, pathogen bacteria which are in a very low number might be under stress or be damaged. Therefore, bacteria might not proliferate in selective media, causing false results. Molecular methods like FISH method are used frequently in order to show the presence of viable but nonculturable bacteria [15]. FISH method is a fast and specific tool in determination of complex microbial population in not only food products, but also in environment like soil and mud [15].

In this study, it was aimed that 50 minced meat specimens obtained from 27 butchers and 23 supermarkets in nine different towns in Istanbul were investigated for the presence of *Salmonella* bacteria by conventional culture method (ISO 6579 reference method), immunomagnetic separation method and fluorescent *in situ* hybridization (FISH) method.

MATERIAL and METHODS

Sampling

In the current study, a total of fifty minced meat samples were purchased from a variety of retail outlets and supermarkets. Samples were transported to the laboratory in a refrigerated container and stored at 4°C until examined.

The Detection of Salmonella spp.

Under aseptic conditions, 25 g samples were taken, homogenised in 225 mL of Buffered Peptone Water (HiMedia, India) in a Stomacher (IUL Instrument, Spain) for 1 min and incubated at 37°C for 24 h [16] for pre-enrichment. Each culture was used for further testing with the three methods.

After incubation, three different methods were used to determine *Salmonella* bacteria. These methods were conventional culture (ISO 6579), immunomagnetc separation (IMS) and fluorescent *in situ* hybridization (FISH) methods.

Conventional Culture Method

Conventional culture method procedures for isolation of *Salmonella* were performed according to the International Organization for Standardization (ISO 6579) [13,17]. An aliquot of 0.1 mL BPW pre-enriched samples was inoculated to 9.9 mL Rappaport-Vasiliadis (RV) broth (HiMedia, India) and incubated at 42°C for 24 h [18]. After 24 h incubation, enriched broth (RV broth) was diluted from 10^{-1} to 10^{-4}. 25 µL of enriched broth was streaked on to Salmonella Shigella (SS) agar (HiMedia, India) and brilliant green phenol red lactose sucrose (BPLS) agar (HiMedia, India) and incubated at 37°C for 24 h. Following 24 h incubation, plates were examined for typical colonies, picking at least one colony of each typical colonial type from each of the plates for identification. Colonies of presumptive *Salmonella* are subcultured, and are confirmed by using API 20E. Serological verification of the *Salmonella* bacteria was made by using commercially available test kits (RTA Laboratories, Gebze).

Immunomagnetic Separation Method (IMS)

In the current study, Dynabeads anti-*Salmonella*

conjugates (Dynal®, Norway) and Dynal® MX3 sample mixer were used [19]. Anti-*Salmonella* paramagnetic beads are anti-*Salmonella* antibodies that bound covalently bacteria surface. The immunomagnetic separation was accomplished starting from 1 mL the pre-enrichment broth was transferred to an Eppendorf tube containing 20 µL of the immunomagnetic microbeads coated with anti-*Salmonella* [20,21]. The tubes were shaken on a Dynal® MX3 sample mixer (Dynal®, Norway) at room temperature for 10 min. Following 10 min. incubation, they placed to a magnetic separator (Dynal®, Norway) [18], and the supernatant was removed from the tubes by Pasteur pipette. 1 mL of phosphate buffer saline (PBS) containing 0.05% of Tween 20 was added to the tube containing the microbeads, the tube was shaken on a Dynal® MX3 sample mixer (Dynal®, Norway) at room temperature for another 3 min. The tubes were placed back in a magnetic separator (Dynal®, Norway). This washing process was repeated 3 times. After washing procedure, 0.1 mL PBS was added to sample and sample was diluted from 10^{-1} to 10^{-4}. Aliquots (25 µL) were streaked on SS agar and BPLS agar. After this step, the procedure was continued as described above, the conventional culture method.

Fluorescent in situ Hybridization (FISH) Method

FISH analysis was carried out to identify *Salmonella* spp. using the VIT-*Salmonella* kit (Vermicon, Munich, Germany). FISH method was applied according to the instructions of manufacturer company as follows:

0.1 mL pre-enrichment sample was transferred to 9.9 mL Rappaport Vassiliadis (RV) broth. After incubation at 42°C for 4-6 h, 2 mL sample was centrifuged at 2.700-4.000 g for 5 min. Then supernatant fluid was removed and 4 drops of 'B2 solution' were added on the sediment. Then 5-10 µL of the prepared sample was added to each well of the slide and the slide was dried horizontally at 46°C for 15-30 min. After incubation, one drop 'Solution B2' was added to each well and it was dried again horizontally at 46°C for 15-30 min. The tank was inserted a small way into the VIT-Reactor. 25 drops of 'Solution C6' was placed around in the tank and then the slide it fully into the VIT-Reactor. 1 drop of 'negative control' (brown), 1 drop of 'VIT (Sal)' (green) and 1 drop of 'positive control' (red) were added to wells. The slide was inserted into the VIT-Reactor carefully and it was dried horizontally at 46°C for 90 min. After incubation VIT Reactor was opened and the slide was removed carefully. 'Solution D6' (washing solution) that was diluted twenty-fold with distilled water, was preheated at 46°C for 30 min. VIT-Reactor was filled with the preheated washing solution. The slide was inserted carefully into the VIT-Reactor and it was closed. The drops were let run into each other and it was incubated at 46°C for 15 min. After 15 incubation, VIT-Reactor was opened and slide and washing solution were removed. VIT-Reactor was filled with distilled water and the slide was inserted into the distilled water and then slide was removed immediately. The slide was dried 46°C for 15 min. A small drop of 'Finisher' was placed between the wells on the slide. The slides were examined under epifluorescent green under the blue light (EX-465-495), and also fluorescent red under the green light (EX-510-560) were evaluated as *Salmonella* and photographs were taken.

Determination of Sensitivities of Conventional Culture and Immunomagnetic Separation Methods

The sensitivities of conventional culture (ISO) and immunomagnetic separation (IMS) method, which are used to detect *Salmonella* bacteria, are calculated by using the formula described in Boer and Beumer [22]:

$$\text{Sensitivity} = \frac{\text{Number of positive samples (P)}}{\text{P + Number of negative samples (FN)}} \times 100$$

If *Salmonella* bacteria can be isolated from the sample, P is the number of true positive samples. If *Salmonella* can not be isolated from the sample, N is the number of true negative samples. When *Salmonella* bacteria can be isolated with at least one method, if the method can not isolate *Salmonella* bacteria, false negative is present and shown with FN.

RESULTS

In the current study, *Salmonella* bacteria in minced meat samples were detected by using conventional culture method, IMS and FISH methods. By using conventional culture method, five of fifty minced meat samples showed *Salmonella* bacteria, while IMS method showed three and FISH showed thirty-seven. 12 out of 50 (24%) minced meat samples did not show any presence of *Salmonella* bacteria with three methods used. Only one sample (sample 30) showed *Salmonella* bacteria by using three methods. *Salmonella* bacteria were detected, by FISH method, in 37 of 50 minced meat samples *(Table 1, Fig. 1)*.

As seen in *Table 1*, conventional culture and immunomagnetic separation methods helped isolation of *Salmonella* bacteria in seven samples. According to the formula described in Boer and Beumer [21], the sensivities of the methods used are found to be as 71.4% for conventional culture method, and 42.8% for IMS. Based on the data and calculations given above, conventional culture method was found to be 28.6% more sensitive than immunomagnetic separation method.

DISCUSSION

With conventional culture method, five of fifty minced meat samples showed the presence of *Salmonella* bacteria. Tekinşen et al.[23] reported that no samples out of 20 ready to use minced meat samples sold in supermarkets in Ankara had *Salmonella* bacteria. The reason why *Salmonella* bacteria did not show in these types of food products is thought to be the presence of some organisms

Table 1. *Comparison of methods used* for the isolation of *Salmonella bacteria*
Tablo 1. *Salmonella cinsi bakterilerin tespitinde kullanılan yöntemlerin karşılaştırılması*

Sample Number	Conventional Culture Method	IMS Method	FISH Method	Sample Number	Conventional Culture Method	IMS Method	FISH Method
1	-	-	+	26	-	-	+
2	-	-	+	27	-	-	+
3	-	-	+	28	+	-	+
4	-	+	+	29	+	-	+
5	-	-	+	30	+	+	+
6	-	-	-	31	-	-	+
7	-	-	+	32	-	-	+
8	-	-	+	33	-	-	+
9	-	-	+	34	-	-	+
10	-	-	+	35	-	-	+
11	-	-	+	36	-	-	+
12	-	-	-	37	-	-	-
13	-	-	+	38	-	-	+
14	-	-	+	39	-	+	+
15	-	-	+	40	-	-	-
16	-	-	+	41	-	-	-
17	-	-	+	42	-	-	+
18	+	-	-	43	-	-	-
19	-	-	+	44	-	-	+
20	-	-	+	45	-	-	-
21	+	-	+	46	-	-	-
22	-	-	+	47	-	-	-
23	-	-	-	48	-	-	+
24	-	-	+	49	-	-	-
25	-	-	+	50	-	-	-

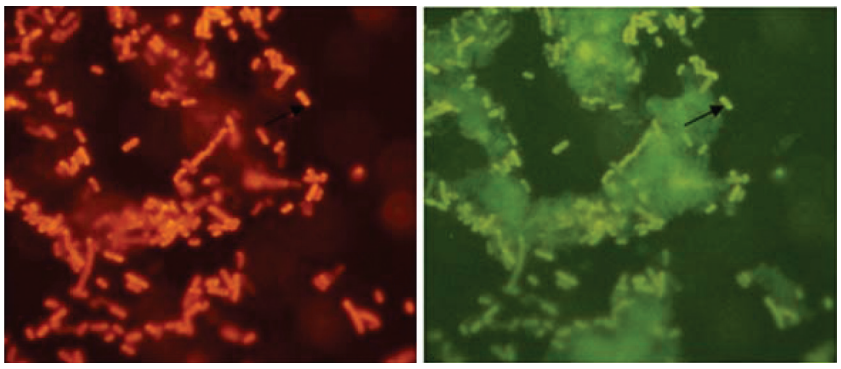

Fig 1. *Salmonella* positive sample by FISH method

Şekil 1. FISH yöntemiyle *Salmonella* pozitif örnek

producing lactic acid, which hampers the production of Gram-negative bacteria dramatically [24]. In another study by Sarıgöl [25], one of twenty minced meat samples obtained from butchers in Elazığ showed the presence of *Salmonella* bacteria, and another study by Gökalp et al.[26] yielded that one of forty-eight samples in Erzurum (obtained from butchers and Meat and Fish Institution) had *Salmonella* bacteria. Aabo et al.[27] found *Salmonella* bacteria in only one specimen out of forty-eight cattle minced meat samples, by using the conventional culture method. Erol [5] reported the isolation of *Salmonella* bacteria from only four of one-hundred-and-twenty samples (3.3%).

Detailed literature surveys showed that the presence of *Salmonella* bacteria examined by IMS method is generally found in chicken [18] and pork, cacao, powdered milk and sausage [8]; however, minced meat samples are found to be investigated in a very low number of publications. In the current study, as an alternative to the conventional culture method, IMS method showed that only three samples out of fifty samples examined had *Salmonella* bacteria. Cudjoe and Krona [13] investigated the presence of *Salmonella* bacteria by conventional culture and IMS methods, and they reported that the cultures obtained with conventional culture method had a more dense growth of competitive

flora. For this reason, it was suggested that the use of IMS method can prevent largely the competitive flora development and this will speed up the selection of suspicious *Salmonella* colonies from selective solid media [13,28]. Although some researchers [13,29] reported that IMS method gave better results than conventional culture method, our study shows that conventional culture method is more effective than IMS method. Jeníková et al.[4] published an article which investigated the use of IMS method for detecting the presence of *Salmonella* bacteria in minced meat samples, and reported that foods with high fat content were not suitable for IMS. They also reported that in a food like minced meat, which has a high fat content, magnetic beads will be lost in food matrix and therefore cannot be acquired by magnetic field, so IMS method is not successful. In such a case, because of competitive microflora (especially, bacteria of *Enterobacteriaceae* family) yield cross-reactions and other bacteria than *Salmonella* can bind to the anti-*Salmonella* Dynabeads. These factors lead to the decrease in sensitivity for IMS method, used to detect *Salmonella* bacteria [4]. Some researchers conclude this failure as both immunomagnetic particles are lost in meat with high fat content and as not using a medium with better selectivity [12,16]. In a study published by Mercanoğlu et al.[20], it was determined that anti-*Salmonella* globules could capture *Enterobacter aerogenes, Escherichia coli* and *Klebsiella pneumoniae* bacteria. It was observed that the bindings which are not specific to anti-*Salmonella* globules of these bacteria did not prevent *Salmonella* bacteria to bind to globules and after IMS application, different type of bacteria can develop with these bacteria in selective solid media [19,30]. In addition, it is thought that the cross reactions might be caused from bacterial strain and environmental conditions [31]. Our study supported the study carried out by Jenikova et al.[4] in which cross-reactions caused by competitive microflora was detected. After the use of IMS method, on the selective medium, *Salmonella* bacteria along with bacteria belonging to *Enterobacteriaceae* family such as *Proteus vulgaris, Proteus mirabilis, Citrobacter freundii, Citrobacter youngea* and *E. coli* were found. In our study, on the contrary to the study by Mercanoğlu et al.[20], as a result of cultivation on BPLS agar after IMS application, it was determined that anti-*Salmonella* globules captured *P. vulgaris* and growth of this bacteria determined on the medium. With conventional culture method and IMS method, it is thought that isolated erroneous positive strains like *C. freundii, Citrobacter brakii, C. youngeae, P. vulgaris, P. mirabilis, Morganella morganii,* and *Hafnia alvei* suppress the growth of *Salmonella* bacteria. Especially, it is considered that bacteria out of *Salmonella*, captured by magnetic particles by IMS method, cause diminisment of the sensitivity of the method.

With IMS method, it is reported that when detergents like Tween 20 or protamin are added to PBS solution, which is used as a washing solution in IMS method, there are reductions in the numbers of non-specific bindings [19,32,33]. In our study, it was found that Tween 20, added in a concentration of 0.05 mL/100 mL, to the washing solution (PBS solution) in IMS method could not prevent cross reactions and that along with *Salmonella* bacteria, other bacteria belonging to the *Enterobacteriaceae* family could grow in the medium. With IMS method, another reason for low recovery percentages of *Salmonella* bacteria might be the first washing procedure which might remove *Salmonella* bacteria with other bacteria [19,30,33]. If the number of *Salmonella* bacteria is greater than anti-*Salmonella* globules, *Salmonella* bacteria might not be captured by globules and with the first washing procedure, bacteria which did not bind to the globules might be washed out. However, even if there is only one *Salmonella* bacterium in the sample, this bacterium can be captured by anti-*Salmonella* globule. Literature survey shows that there is a few number of publications about investigation of *Salmonella* bacteria in minced meat. Erol [5] reported that *Salmonella anatum, Salmonella telaviv, Salmonella typhimurium* were isolated from minced meat samples, whereas Fratamico [34] reports that *Salmonella cerro, S. typhimurium, S. anatum* and *Salmonella infantis* were isolated from minced meat samples. In the study by Gökmen and Alişarlı [35], *Salmonella* spp. were isolated from minced meat samples in Van. In our study, conventional culture method yielded *Salmonella* spp. and *S. choleraesuis ssp. arizonae* to be isolated in five of fifty samples.

In our study, the presence of *Salmonella* bacteria was searched by conventional, IMS, and also by FISH method. Our study showed that conventional culture method yielded *Salmonella* bacteria in 5 samples out of 50 total while FISH method found this bacteria in 37 samples out of 50 total. Literature survey showed that with FISH method, *Salmonella* bacteria were searched generally in pork [17,36]. In a publication by Vieira - Pinto et al.[36], 16 out of 47 samples yielded the presence of *Salmonella*-type bacteria in pork samples. In another study it was determined that FISH method gave better results than conventional culture method [17]. The reason why FISH method gives better results than conventional culture method is considered to be due to the presence of actually dead or viable but non-culturable *Salmonella* bacteria for several stress factors [17,37]. In FISH method, these viable but non-culturable cells can show metabolic activities with their ribozomes in low number. However, FISH method is not affected from physical and chemical properties of food (temperature, salt concentration and pH), which cause *Salmonella* cells to undergo stress and cause bacteria not to be cultured. FISH method is more advantageous because a) it is not affected from inhibitory factors, b) it uses less material than conventional culture method and c) it is faster.

FISH method can detect *Salmonella* bacteria among high number of competitive microflora. However, in sample18, conventional culture method was successful in detecting

Salmonella bacteria but FISH method failed. The possible cause being the presence of a low amount of bacteria within the scanned area by microscope [40]. In addition, erroneous negative result might be due to experimental errors.

Time is a very important factor in detecting the pathogens in foods and for this reason, it is needed to use new methods which can give results in a short time. However, fast detection of epidemics due to food contaminated with pathogens is important, but sources of bacteria and obtaining bacterial isolates are more important. For this reason, the results obtained with new methods in short time must be compared with and supported by the gold standard, that is, conventional culture method.

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