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Evaluation of the EF-18 Agar-Hydrophobic Grid Membrane Filter (HGMF) Method to Isolate *Salmonella* from Poultry Products

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The EF-18 agar/hydrophobic grid membrane filter (EF18/HGMF) method was evaluated for the isolation of *Salmonella* in naturally contaminated chicken carcasses, chicken parts (legs, wings and giblets) and processed chicken products (sausages and hamburgers). Percentages of false positive results for *Salmonella* (colonies with a similar morphology to those of *Salmonella*) were 78.75, 81.67 and 80% for carcasses, chicken parts and processed chicken products, respectively. The bacterial isolates that caused false positive reactions using this method were identified as *Proteus mirabilis* (70.85%), *Citrobacter freundii* (15.25%), *Klebsiella ozaenae* (5.83%), *Hafnia alvei* (4.48%), *Escherichia coli* (2.69%) and *Enterobacter aerogenes* (0.90%). The data obtained in this study suggest that the EF-18/HGMF method is not sufficiently selective or specific for isolating *Salmonella* from meat and chicken products.

Key words**:** *Salmonella*, poultry products, medium, EF-18, HGMF

Salmonellae are recognized as the leading cause of bacterial gastroenteritis in humans (6). Foodborne infections with members of the genus *Salmonella* cause considerable morbidity, mortality, and economic burdens, and may be specially severe in the very young, the elderly, and the immunocompromised.

Poultry products are considered to be the single most prevalent source of *Salmonella* among all animal-derived food products (2). Poultry meat can be a vehicle of foodborne salmonellosis when the raw product initially contaminated with *Salmonella* cells is delivered to the consumer or due to subsequent undercooking, cross-contamination, or improper thawing (4).

The high morbidity associated with human salmonellosis has led to the development of several methods and culture media for the detection of salmonellae in food products (17, 18). A rapid *Salmonella* detection method, based on the use of EF-18 agar in conjunction with the hydrophobic grid membrane filter (EF18/HGMF) method (QA Life Sciences, Inc.) was introduced in 1982 (11). This method was accepted as an AOAC official action in 1984 (8), accorded First Action Status by AOAC in 1990 (9) and recommended by APHA (13). However, various investigators have obtained conflicting results when evaluating this method. The objective of this study was to evaluate the selectivity and specificity of the EF-18/HGMF method for isolation of *Salmonella* in naturally contaminated meat and chicken products.

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Materials and Methods

Samples

Forty chicken carcasses, fifteen chicken parts (5 legs, 5 wings and 5 giblets, livers and hearts) and fifteen processed chicken products (5 red sausages, 5 white sausages and 5 hamburgers) were purchased from different retail outlets in León (north-west Spain). Each sample was placed in a separate sterile plastic bag. Samples were transported on ice to the laboratory immediately after collection and tested within four hours.

Microbiological analyses

Twenty-five grams of sample was removed from each product using a sterile scalpel and placed into a sterile stomacher bag containing 225 ml of nutrient broth (NB, Oxoid Ltd., Hampshire, England). For products containing skin (carcasses, legs and wings) 25 g of the skin was excised as a sample. For carcasses the 25 g was made up of samples of breast, wing, leg, neck, dorsal and ventral skin. Skin samples and NB were homogenized in a stomacher (Stomacher 400, A.J. Seward, London, England) for 2 min and incubated in the bag for 24 h at 35°C. After incubation, 0.1 ml of the preenriched sample was diluted in 10 ml of pre-tempered tetrathionate brillant green (TBG, QA Life Sciences, San Diego, CA, USA). After incubation for 6-8 h in a 35°C waterbath, serial decimal dilutions were carried

out in 0.1% (w/v) sterile Peptone Water (Oxoid Ltd., Hampshire, England), and 1 ml of the 10^{-2} dilution was filtered through an Iso-Grid hydrophobic grid membrane filter (HGMF) with the aid of a sterile Iso-Grid filtration unit (QA Life Sciences, San Diego, CA, USA) and a vacuum pump. Filters were aseptically transferred using forceps to the surface of EF-18 agar (QA Life Sciences, San Diego, CA, USA) plates and incubated for 24 h at 42°C.

Identification of isolates

Four green, blue-green or blue colonies (typical *Salmonella* colonies exhibiting a lysine-positive and sucrose-negative reaction) were selected from plates for each chicken sample for subsequent characterization on the basis of the following tests: Gram stain (14), catalase reaction, oxidase reaction, oxidation/fermentation of glucose, motility test, nitrate reduction (5), sulphide indol motility (SIM), phenylalanine (phe) deaminase (15), triple sugar iron (TSI) agar, lysine iron agar (LIA), citrate, methyl red, Voges Proskauer, urease, ornitine (orn) and lysine (lys) decarboxylase, arginine (arg) hydrolase (12) and β-galactosidase (1). The strains were grouped in biochemical profiles according to the results in the previous tests. The identification was performed following the identification schemes proposed by Brenner (3) and Ewing (12). Moreover, in order to confirm the identifications carried out, one strain from each profile was inoculated into microtubes of API 20E strips (bioMérieux S.A., Marcy-l'Étoile, France) according to the manufacturer's instructions. The bacteria were identified using the database provided by the manufacturer (API LAB Plus ver 3.2.2.).

Results and Discussion

In order to measure the specificity of the presumptive positive results produced by the EF-18/HGMF method, we monitored the confirmation ratio of presumptive-positive samples (samples with typical colonies on EF-18 agar). As was previously indicated, four typical colonies were identified for each sample analyzed. Results are shown in Table 1. Thirty-one samples (47.69% of samples with presumptive *Salmonella* colonies) were identified as false-positives after confirmation tests. Percentages of false positive samples were similar in the different types of products (45% for chicken carcasses, 50% for chicken parts and 53.85% for processed chicken products). These percentages are much higher than ratios obtained by Entis and Boleszczuk (10) who found that only three of the 954 (0.3%) samples of naturally and artificially contaminated food categories were false positives. When the authors used a "conventional" method for isolation, the number of false positives increased to 75 (7.9%). When the foodstuff studied was raw ground poultry and poultry carcasses (naturally contaminated) no false positives were detected.

The numbers and percentages of false positive colonies (colonies of other microorganisms with a similar morphology to *Salmonella*) are shown in Table 2. Of the 280 presumptive salmonellae isolated, only 57 (20.35%) were identified as *Salmonella*. The remaining 223 strains (79.65%) were false positives. Previous studies carried out using the EF-18/HGMF method had shown percentages of false positives much lower than those presented here. Entis (7) found that only 21 of the 763 strains (2.75%) isolated from 285 raw chicken meat samples were false positives. Sharpe (18) obtained a lower percentage of false positives: 0.5%.

As the data in Table 3 indicate, the majority of the false positive colonies (70.85%) were *Proteus mirabilis*. Other species detected were *Citrobacter freundii* (15.25%), *Klebsiella ozaenae* (5.83%), *Hafnia alvei* (4.48%), *Escherichia coli* (2.69%) and *Enterobacter aerogenes* (0.90%). Warburton *et al.* (21) when analysing different naturally and artificially contaminated foods (including naturally

Table 1. Samples with presumptive *Salmonella* colonies on EF-18 agar and *Salmonella* false positive samples

Chicken product	Samples analyzed	Samples with typical colonies (presumptive *Salmonella*)	Samples without confirmed *Salmonella* (*Salmonella* false-positive samples)
Carcasses	40	40	18 (45%)
Chicken parts	15	12	6 (50%)
legs	5	4	2 (50%)
wings	5	4	2 (50%)
giblets	5	4	2 (50%)
Processed chicken products	15	13	7 (53.85%)
red sausages	5	5	3 (60%)
white sausages	5	5	2 (40%)
hamburgers	5	3	2 (66.66%)
Total	70	65	31 (47.69%)

Table 2. Number (percentage) of true positive (*Salmonella*) and false positive reactions on EF-18 agar

Chicken product	Presumptive salmonellae isolated	Number (percentage) of salmonellae confirmed	Number (percentage) of false positives
Carcasses	160	34 (21.25%)	126 (78.75%)
Chicken parts	60	11 (18.33%)	49 (81.67%)
legs	20	3 (15%)	17 (85%)
wings	20	5 (25%)	15 (75%)
giblets	20	3 (15%)	17 (85%)
Processed chicken products	60	12 (20%)	48 (80%)
red sausages	20	4 (20%)	16 (80%)
white sausages	20	6 (30%)	14 (70%)
hamburgers	20	2 (10%)	18 (90%)
Total	280	57 (20.35%)	223 (79.65%)

Table 3. False positives isolated from chicken products

Chicken product	Total	Number (percentage) of false positive colonies					
		Proteus mirabilis	*Citrobacter freundii*	*Klebsiella ozaenae*	*Hafnia alvei*	*Escherichia coli*	*Enterobacter aerogenes*
Carcasses	126	114 (90.48)	2 (1.58)	2 (1.58)	-	6 (4.76)	2 (1.58)
Chicken parts	49	19 (38.78)	16 (32.65)	10 (20.41)	4 (8.16)	-	-
legs	17	5 (29.41)	7 (41.18)	5 (29.41)	-	-	-
wings	15	6 (40)	4 (26.67)	5 (33.33)	-	-	-
giblets	17	8 (47.06)	5 (29.41)	-	4 (23.53)	-	-
Processed chicken products	48	25 (52.08)	16 (33.33)	1 (2.08)	6 (12.50)	-	-
red sausages	16	6 (37.5)	9 (56.25)	1 (6.25)	-	-	-
white sausages	14	10 (71.43)	-	-	4 (28.57)	-	-
hamburgers	18	9 (50)	7 (38.89)	-	2 (11.11)	-	-
Total	223	158 (70.85)	34 (15.25)	13 (5.83)	10 (4.48)	6 (2.69)	2 (0.90)

contaminated poultry), also detected *C. freundii*, *E. aerogenes*, *E. coli* and *H. alvei* among the microorganisms giving false positive reactions on EF-18 medium. The appearance on this culture media of *Enterobacteriaceae* colonies other than *Salmonella* with a similar morphology to those of *Salmonella* was also found by Sharpe (18). This author streaked 282 strains of *Enterobacteriaceae* on EF-18 agar, and found that 31 (11%) of them, including 22/120 *E. coli* strains, caused colonies similar to those of *Salmonella*.

The high percentage of false positives that we have identified as *P. mirabilis* is not unusual for a number of reasons. Firstly, this bacterium frequently contaminates poultry meat and it is highly resistant to the selective agents normally used in selective media to isolate *Salmonella* and other *Enterobacteriaceae* (Capita, unpublished data). Moreover, the morphology of *P. mirabilis* on EF-18 agar is similar to that of the *Salmonella* colonies since they generally are unable to ferment the sucrose (12). The fact that *P. mirabilis* exhibits a lysine decarboxylase negative reaction gives them a slightly greener color than that of the *Salmonella* colonies, which, from our point of view, makes both microorganisms difficult to distinguish. It must be pointed out that a wide variation in the coloration of *Salmonella* may be observed even when pure stock cultures were used as controls (20). This variance makes distinction between *P. mirabilis* and *Salmonella* colonies difficult. None of the remaining strains isolated fermented the sucrose and decarboxylated lysine, which indicates that their morphology is similar to that of the *Salmonella* colonies. This fact would explain that they were isolated as presumptive salmonellae. However, it must be pointed out that according to Entis and Boleszczuk (10), most competitors including *Proteus* spp., *Citrobacter* spp., *Enterobacter* spp., *Klebsiella* spp. and many *E. coli* strains produce yellow colonies and not green, blue-green or blue colonies.

When evaluating EF-18 medium to determine the percentage of false negatives, Entis (7) found a low percentage of only 1.4%. Sharpe (17), with the EF-18/HGMF method found 1.94% false negatives. Other authors, however, found higher percentages of false negatives. Warburton *et al.* (21) show that this agar did not readily differentiate atypical sucrose positive salmonellae. When comparing the EF-18 method with a reference (Health Protection Branch) method, between 5.9% and 8.3% false negatives for artificially contaminated foods, and between 17.5% and 43.5% for naturally contaminated foods (20) were obtained. These authors indicate that the high percentages of false negatives is due to the large number of other competing microorganisms in the selective broth, which hinders the isolating of *Salmonella* colonies. According to these researchers, EF-18 agar is viewed negatively in that it produces small colonies on HGMFs, may cause overgrowth by competitors, and the wide variations in the blue/green coloration of *Salmonella* colonies. The high percentages of false negatives associated with this method can limit its use as a screening test. Some authors report that many of the problems with this method are due to the use of filters. When evaluating streak and spread plates (not using HGMFs), EF-18 has been shown to be a suitable medium for isolating *Salmonella*, and comparable to other selective agars (19, 20) or even better (16). Nevertheless, it must be taken into account that in the absence of HGMFs, contaminating microorganisms capable of growing in the EF-18 medium can form large colonies which hide the salmonellae present. In our study neither the percentages of false negative samples nor the false negative colonies were determined.

The data obtained in this study suggest that the EF-18/HGMF method is not sufficiently selective or specific for isolating *Salmonella* from chicken meat products. These foods are highly contaminated with other microorganisms, many of which are resistant to the selective agents of the enrichment broth and culture agar of this method. Moreover, some of these microorganisms are capable of forming colonies with a similar coloration to those of *Salmonella* on EF-18 agar, which causes the high level of false positives.

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