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A Comparison of EF-18 Agar and Modified Brilliant Green Agar with Lutensit for Isolation of *Salmonella* from Poultry Samples

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Petersen, L.: A comparison of EF-18 agar and modified brilliant green agar with lutensit for isolation of *Salmonella* from poultry samples. Acta vet. scand. 1997, 38, 79-85. – Two selective plating media were compared to determine their performance in a three-step protocol for isolation of *Salmonella* from faecal, litter, and fluff samples. The encichment protocol consisted of: 1) Preenrichment in buffered peptone water (BPW), 2) Selective enrichment in Rappaport-Vassiliadis broth, and 3) Plating onto EF-18 agar and BGA/L simultaneously.

From a total of 1101 samples, *Salmonella* was isolated from 158, 157 of which were faecal samples. Thirtyone of these isolates were recovered on one medium only, 18 could not be found on BGA/L and 13 could not be found on EF-18 agar. The relative specificity and sensitivity of each plating agar was determined by enumeration of false-positive and false-negative reactions. EF-18 agar compared favourably with BGA/L, displaying a sensitivity of 0.92 as opposed to 0.89 for BGA/L, calculated for the "fecal samples" group only. The calculated specificities for each group of samples were likewise considerably higher for EF-18 agar (0.75-0.91) than for BGA/L (0.35-0.55). Though EF-18 agar is slightly more expensive than BGA/L, the routine use of the former may result in a considerable reduction in overall laboratory costs due to its superior selectivity. On the other hand the combination of the 2 media clearly would reduce the number of false negative results, with little extra cost.

detection; selective and indicative agar; novobiocin; sulfapyridine.

Introduction

Multiple methods exist for the determination of *Salmonella* contamination of a sample, including culturebased, immunological and genetic procedures. The investigation of spread of *Salmonella* infection among poultry flocks depends very much on the availability of suitable detection methods. Some epidemiological markers that have proven very useful (serotyping, phagetyping etc.) can only be determined if a culture of the given strain exists, therefore culturebased methods inheritantly give some unique opportunities.

Microbiological analysis for *Salmonella* generally includes an enrichment step in a selective broth medium (typically selenite, Rappaport-Vassiliadis, or tetrathionate broth), often preceded by a nonselective preenrichment step that allows injured cells to recover. The enrichment broth is streaked on one or 2 selective agar media, and presumptive positive colonies are verified biochemically and serologically.

The choice of selective agar media for *Salmonella* analysis has been the subject of several recent studies (*Sherrod et. al.* 1995, *Warburton et*

al. 1995). The type of sample, and especially the composition of the background flora, is of considerable importance for the efficiency of a specific plating medium. The traditional selective plating media (such as Brilliant Green Agar, Xylose Lysine Desoxycholate agar and others) in fact allow a broad selection of especially Gram negative bacteria to grow on the agar plates. This trait hinders the examination of samples such as faecal and environmental samples which are heavily contaminated with Gram negative bacteria. Growth of non-salmonellae may disturb the reading of plates, because well isolated colonies of *Salmonella* may not be obtained. In our laboratory we have experienced that some *Enterobacteriaceae,* for instance *Proteus* spp. and *Escherichia* spp., produce "*Salmonella*-like" colonies on BGA and XLD, which may also to some degree agglutinate with polyvalent antisera (*Kauffmann* 1954). Furthermore, it is possible that colonies of *Salmonella* on selective plates in some instances may be missed because of overgrowth by background flora that masks typical reactions.

Several newly developed plating agars (Rambach, EF-18 etc.) have been reported to compete well with the classical media, especially due to well performing visualization systems, that make it simple to distinguish between *Salmonella* and background flora (*Joosten et al.* 1994, *Sherrod et al.* 1995).

EF-18 agar is a relatively cheep plating medium, which at the same time is reported to simplify the recognition of *Salmonella.* In a collaborative study in Canada it proved to perform very well for routine analysis compared to Rambach agar and XLD among others, also in laboratories that had not used EF-18 before (*Warburton et al.* 1995).

BGA agar, modified by addition of the detergent lutensit (BGA/L), has been in use in our laboratory for routine purposes for several years. Though the staff has achieved considerable expertise in using the medium, the "background flora" factor is time and ressource consuming, since it is necessary to handle a considerable number of presumptive positive colonies every day.

The purpose of this study was to compare BGA/L with EF-18 agar in order to explore the specificity and sensitivity of the 2 media.

Materials and methods

Collection and analysis of samples

The study material comprised 1,101 individual samples: 133 samples of hatchery dust and egg shells (fluff), 257 samples of litter material from breeder flocks, and 711 faecal samples from broiler flocks.

Hatchery samples, consisting of dust and egg shell material (25 g), were collected from each hatcher after hatching, before cleaning and disinfection procedures.

Litter samples were collected from breeder flocks every week (10 g of sample).

Faecal samples from broiler flocks were collected 2-3 weeks prior to slaughter. Twelve samples (pools of 5, 10 g of sample) were collected from each flock randomly distributed throughout the house.

The samples were mailed to the laboratory. Samples that were received the day before or during a weekend were stored at 4 °C upon receipt at the laboratory.

All samples were analysed by a common enrichment procedure: Preenrichment was carried out in phosphate buffered peptone water (BPW) as described in the NMKL procedure (*Anon.* 1991). 10 g of sample was preenriched in 90 ml BPW, or 25 g of sample was preenriched in 225 ml BPW, for 18-24 h at 37 °C. 0.1 ml of the preenrichment culture was transferred to 9.9 ml Rappaport-Vassiliadis (RV) broth (Oxoid CM 669) and incubated for 18-24 h at 37 °C (*Anon.* 1991).

Agar media

RV enrichment cultures were plated in parallel onto BGA/L and EF-18 agar by means of a 10μl loop.

BGA/L: Brilliant green agar (Oxoid CM 329), modified by the addition of Lutensit A-LBA (BASF) 0.15% v/v from the medium described by *Bisgaard* (1978). Plates were incubated for 18-24 h at 37 °C.

EF-18-agar plates were prepared according to *Entis* (1990) with the following: 5.0 g proteose peptone, 3.0 g yeast extract, 10.0 g L-lysine monohydrochloride, 2.5 g D-glucose, 15.0 g sucrose, 1.5 g $MgSO_4.7H_2O$, 1.5 g bile salts, 0.3 g sulfapyridine, 0.03 g bromothymol blue sodium salt, and 15.0 g agar, 0.015 g novobiocin, 1000 ml H_2O; pH 6.6 ± 0.1. Plates were incubated for 18-24 h at 42 °C.

Verification of isolates

Typical colonies on EF-18 agar and BGA/L agar were verified by serotyping according to the Kauffmann-White-Schema (*Kauffmann* 1972) using polyvalent antisera produced by Statens Serum Institut, Copenhagen, DK.

At least 2 red colonies were picked from BGA/L, and 2 blue colonies from EF-18 plates. From certain plates harbouring many colonies of slightly different morphology but typical color, the number of colonies picked could be larger.

Data evaluation

Results obtained by plating on the 2 media were tabulated against the "true" *salmonella* status of the sample. The true status was defined as *Salmonella* positive if Salmonella was isolated from a sample by at least one plating medium, if not as *Salmonella* negative.

The sensitivity of each plating medium was calculated as the number of true *Salmonella* positive samples detected by that plating medium ($N_{posMedium}$), divided by the total number of true

Table 1. Comparison of EF-18 agar and BGA/L for the isolation of *Salmonella* spp. from poultry samples after selective enrichment in RV broth.

	Total number	True positive[a]			Negative[b]	False negative[c]		False positive[d]		Identified negative[e]		Sensitivity/specificity of media	
	N	$N_{posTotal}$	$N_{posEF-18}$	$N_{posBGA/L}$	N_{neg}	EF-18	BGA/L	EF-18	BGA/L	$N_{negEF-18}$	$N_{negBGA/L}$	EF-18	BGA/L
Faecal samples	711	157	144	139	554	13	18	48***	359	506	195	0.92/0.91	0.89/0.35
Litter samples	257	1	1	1	256	0	0	58***	129	198	127	nd[f]/0.77	nd /0.50
Fluff samples	133	0	0	0	133	0	0	33***	60	100	73	nd /0.75	nd /0.55
Total	1101	158	145	140	943	13	18	139	548	804	395		

*** Significantly ($p<0.001$) lower number of false positives than BGA/L. [a]Samples that resulted in positive isolation of *Salmonella*. [b]Samples that did not result in positive isolation of *Salmonella*. [c]True positive samples that were not recorded using one medium. [d]Samples resulting in presumptive positive colonies that could not be verified by serotyping as *Salmonella*. [e]Negative samples that did not result in presumptive positive colonies on plates. [f]Not done, due to insufficient number of positives in sample groups.

Table 2. Serovar distribution of 158 Salmonella isolates

Serotype	Number of isolates/percent
Isolates from faecal samples:	
S. 4,12:b:-	1 / 0.6%
S. 6,7:-:-	1 / 0.6%
S. Tennessee	1 / 0.6%
S. Havana	2 / 1.3%
S. Indiana	21 / 13.4%
S. Infantis	27 / 17.2%
S. Typhimurium	104 / 66.2%
Isolate from litter samples:	
S. 4,12:b:-	1 / 100%
Isolates recovered on one medium only:	
Not recovered on BGA/L:	
- *S.* Tennessee	1 / 5.6%
- *S.* 6,7:-:-	1 / 5.6%
- *S.* Infantis	2 / 11.1%
- *S.* Havana	2 / 11.1%
- *S.* Typhimurium	12 / 66.7%
Total	18 / 100%
Not recovered on EF-18:	
- *S.* Typhimurium	13 / 100%

positive samples found (N_{pos}).

The specificity of each plating medium was calculated as the number of samples immediately classified as *Salmonella* negative by that medium ($N_{negMedium}$, samples that were negative, and that did not result in presumptive positive colonies on plates), divided by the total number of negative samples (N_{neg}).

The difference between the number of 'presumptive positive' and 'confirmed positive' samples are referred to as 'false positive' in the text.

For statistical analysis Chi-square analysis and McNemar's test for paired comparisons were used (*Cochran* 1950).

Results

A total of 158 positive samples were detected in this study, the majority of the positive samples being faecal samples from broiler flocks (157 isolates; Table 1). The predominant serovar among the broiler isolates was *S.* Typhimurium (66.2%), followed by *S.* Infantis (17.2%) (Table 2). Only one positive (*S.* 4,12:b:-) was found among the litter samples; all the fluff samples were found negative for *Salmonella.*

Of the 157 faecal isolates, 31 were recovered on one plating medium only, 18 of these on EF-18 agar, and 13 on BGA/L (Table 2). Overall EF-18 agar recovered 144 isolates and BGA/L 139, corresponding to calculated sensitivities of 0.92 for EF-18 and 0.89 for BGA/L (Table 1). As only one positive sample was found among the litter and fluff samples sensitivities were not calculated for these samples.

The serovars of isolates recovered only on EF-18 or BGA/L media are listed in Table 2 together with the serovar distribution of all 158 isolates. It is characteristic that the serovar distribution among the isolates found only on EF-18 was consistent with the distribution among the 157 faecal isolates, while the isolates found only on BGA/L were all Typhimurium. The difference in numbers of positives detected on each medium or in serovar distribution among the isolates recovered on one medium only was not statistically significant, however.

The calculated specificities of the media for each group of samples are listed in Table 1. The specificities of EF-18 are considerably higher than those of BGA/L.

The difference between the 2 media in rates of false positives is highly significant using McNemars test ($p<0.001$) for all groups of samples (Table 1). The McNemar test excludes from comparison the number of samples being false positive on both media. This group of identical samples amount to, for faecal, litter and fluff samples 33, 27 and 14, respectively.

Discussion

Ideally a selective medium for *Salmonella* detection combines a high sensitivity with a high specificity.

The salmonella analysis applied in this study consists of 3 steps. The second and third step have the objective of preventing growth of background flora, so that *Salmonella* can multiply. The third step (plating) is also indicative, allowing *Salmonella* to develop colonies that are characteristic in color and morphology. All 3 steps contribute to the development of the colonies that are ultimately seen on the selective plates. The specificities and sensitivities, computed as described by *Bager & Petersen* (1991), in fact refer to the entire 3 step procedure, even though only the third step is different among procedures. More than one characteristic of the plating medium contribute to the sensitivity and specificity. The selectivity of the medium is important to prevent overgrowth of the plates by background flora, and the visualization system is supposed to make the final distinction between *Salmonella* and the remaining background flora. The interplay between the selective enrichment and the plating medium ultimately determines the efficiency of the entire procedure.

Computing sensitivity and specificity by this procedure for the analysis of naturally infected samples, it is intrinsically assumed that a sample containing *Salmonella* will be detected on at least one medium. That is not necessarily so. For the reasons outlined above the absolute sensitivity and specificity of EF-18 agar and BGA/L remain obscure, whereas the computed values are useful measures of the relative efficiency of the 2 media in this particular procedure.

The relative specificitiy and sensitivity of EF-18 agar are quite promising. In all 3 groups of samples the difference in false positive reactions between the 2 plating agars is highly significant and in favour of EF-18 agar. It was obvious from the examination of the plates that EF-18 agar offers better discrimination between *Salmonella* and background flora. It was often observed when *Salmonella* negative samples were analyzed that EF-18 plates were sterile or had a few yellow colonies, while BGA/L plates were generally covered with varying populations of red and yellow colonies. On the other hand a positive sample typically resulted in heavy growth of blue-green colonies on EF-18.

The selectivity of EF-18 agar is based on bile salts, novobiocin and sulfapyridine, and an incubation temperature of 42 °C. Novobiocin inhibits *Proteus sp.,* which is a major problem in *Salmonella* isolation. The visualization system (L-lysine monohydrochloride, sucrose and bromothymol blue) theoretically allows only some rare strains of enterobacteriaceae, particularly some *Escherichia coli* strains, to develop typical colonies: Blue, blue-green or jadegreen (lysine-positive and sucrose-negative reaction) colonies, slightly irregular, but neither watery nor mucoid (*Brenner* 1984, *Entis* 1990).

BGA/L relies on brilliant green as the selective principle and a lactose/sucrose/phenol red indicator system. Non-lactose/sucrose fermenting organisms grow as red to pink colonies surrounded by brilliant red zones. Salmonella colonies often become bluish and slightly irregular. According to our observations some *Proteus* and *Pseudomonas* spp. grow as red colonies on BGA/L plates, not unlike *Salmonella* colonies. A similar phenomenon is described by the producer of BGA (*Anon.* 1990)

Some members of the family *Enterobacteriaceae* might give moderate reactions with polyvalent antisera (*Kauffmann* 1954). A great number of such cases will cause delay in the diagnostic work, but a certain level can hardly be avoided, and the scarce amount of false positives encountered on EF-18 in this study will in

our mind be acceptable to a routine laboratory. It is characteristic that the media differ in their specificity pattern. EF-18 is more specific for faecal samples than for litter or fluff samples, while BGA/L is more specific for fluff samples, though still not as specific as EF-18 agar.

In conclusion, the results obtained in this study indicate that EF-18 agar indeed is a highly selective plating medium, and that it supports growth of poultry isolates of *Salmonella* at least as well as BGA/L. Ef-18 recovered more isolated than BGA/L, though the difference was not statistically significant. The fact that EF-18 gives rise to considerably fewer false positives than BGA/L will result in a reduced workload and reduced ressource consumption in the laboratory, if only one plating medium is used.

The results obtained in this study underline the importance of streaking enrichment cultures on 2 different plating media. It can be expected that the simultaneous use of both media will improve the efficiency of analysis in the sense that more positives will be identified with little extra cost and effort.

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Sammendrag

En sammenligning af EF-18 agar og modificeret brilliantgrøntagar med lutensit til isolaton af Salmonella *fra fjerkræprøver.*

To selektive plademedier, EF-18 agar og BGA/L blev sammenlignet med hensyn til deres effektivitet. Prøver blev præopformeret i BPW og selektivt opformeret i Rappaport-Vassiliadis bouillon, og derefter spredt på EF-18 agar og BGA/L. Prøvematerialet var fæcesprøver (711), strøelsesprøver (257) og støv- og skalprøver (133) fra slagtekyllinge- og rugeægsproduktion.

EF-18 agars selektivitet er baseret på novobiocin, galdesalte og sulfapyridin. Det indikative system består af L-lysin monohydrochlorid, sakkarose og bromthymolblåt. *Salmonella* vokser på mediet med blå, blågrønne eller jadegrønne kolonier, som er let uregelmæssige, men ikke vandige eller mucoide.

Af i alt 1101 prøver blev *Salmonella* isoleret fra 158,

hvoraf de 157 var fæcesprøver. 31 af isolaterne kunne kun isoleres fra ét plademedium: 18 isolater blev kun fundet på EF-18 agar, og 13 isolater kun på BGA/L. Sensitiviteten af de to medier blev på grundlag heraf beregnet til 0.92 for EF-18 agar, og 0.89 for BGA/L. Der var en signifikant højere forekomst af 'falsk positive' kolonier på BGA/L end på EF-18 agar. De beregnede specificiteter for hver prøvetype var således betydeligt højere for EF-18 agar (0.75-0.91) end for BGA/L (0.35-0.55).

Resultaterne viser at EF-18 agar er meget velegnet til rutineanalyser af prøver fra fjerkræproduktion. Mediets selektivitet og indikative system gør det muligt at diskriminere effektivt mellem *Salmonella* og baggrundsflora.

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