Distinctive Culture Expressions of Enterobacteria Interfering With Isolation of *Salmonella* spp. During the Application of the Recommended ISO 6579–1:2017

Grammato Evangelopoulou , Angeliki R Burriel , Nikolaos Solomakos *

Posted Date: 12 December 2023

doi: 10.20944/preprints202312.0762.v1

Keywords: salmonella; isolation; ISO 6579-1:2017; pre-enrichment; selective media; culture methods; swine

Article

Distinctive Culture Expressions of Enterobacteria Interfering with Isolation of *Salmonella* spp. during the Application of the Recommended ISO 6579-1:2017

Grammato Evangelopoulou [1], Angeliki R Burriel [2] and Nikolaos Solomakos [1,*]

[1] Laboratory of Hygiene of Foods of Animal Origin, Faculty of Veterinary Medicine, University of Thessaly, 224 Trikalon Street, 43100 Karditsa, Greece; matinavet@hotmail.com; nsolom@uth.gr
[2] Department of Nursing, University of Peloponnese, Sparta, Greece; aburriel@uop.gr
* Correspondence: nsolom@uth.gr Tel.: (+306972991083)

Abstract: The objective of the present report is the dissemination of information acquired during the application of ISO 6579-1:2017 for the isolation of *Salmonella* spp. from swine samples. ISO 6579-1:2017 is the officially recommended by the EU protocol for the isolation of *Salmonella* spp., aiming in the harmonization of effective successful control of *Salmonella* infection in food producing animals. Successful control of animal salmonellosis is highly dependent on the sensitivity of the biochemical methods reliably detecting the presence of the pathogen in various stages of food production. Application of ISO 6579-1:2017 resulted in the isolation of twelve *Salmonella* spp. and eighteen other members of the family of Enterobacteriaceae biochemically and antigenically resembling salmonellae. The findings necessitated in the depth evaluation of the culture media xylose-lysine-desoxycholate agar (XLD), Salmonella-Shigella Agar (SS), Brilliant Green Agar (BGA), Salmonella Chromogenic Agar (SCA), Triple Sugar Iron (TSI) and modified semi-solid Rappaport–Vassiliadis (MSRV) agar. The evaluation showed that these culture selective media differed significantly in their performance regarding the isolation of *Salmonella* from swine samples. It was concluded that the presence of atypical *Salmonella* strains negatively affects the prevalence of *Salmonella*, thus the identification of carrier pigs, eventually affecting the efficiency of control programs. Thus, doubtful results require additional biochemical testing for confirming the accuracy of such universally recommended isolation methods.

Keywords: *Salmonella*; isolation; ISO 6579-1:2017; pre-enrichment; selective media; culture methods; swine

1. Introduction

Salmonella spp., are the most prevalent species of pathogens implicated in food-borne infections [1,2]. Thus, their monitoring and control in animals across the EU is a priority. Pigs are an important source of human *Salmonella* spp. infection [3,4], hence pig farms are targets for the control of the pathogen. Its effective control depends on the accurate estimation of *Salmonella* prevalence in a pig population, thus isolation of the agent [5,6] using a combination of culture media. Some of them are, due to their selectivity and sensitivity, the 'gold standard' of *Salmonella* isolation. However, for ensuring protection of the public, a combination of methods is recommended by accreditation bodies and regulatory authorities, such as the International Standards Organization (ISO). *Salmonella* spp. are a genus requiring more techniques and methods for isolation and identification of its members than most other agents of public health importance [7]. The complexity of the investigation of animal salmonellosis demanded a universally applied methodology for the isolation of *Salmonella* spp. from animals and their products across the EU. This methodology is described in EN-ISO 6579-1:2017 [8], becoming the reference method for assessing the prevalence of *Salmonella* spp. infection in fattening and breeding pigs [9–11]. The EN-ISO 6579 has been found to perform better than commercial polymerase chain reaction (PCR), the BAX method (a real time PCR assay) and three other

 doi:10.20944/preprints202312.0762.v1

commercially available systems based on enzyme-linke dimmunosorbent assay [12]. The use of this ISO, although expensive and laborious, is considered a 100% specific method when used in combination with serotyping [13]. As a universally used method, it also helps the comparison of results across the EU, thus the coordination of control programs undertaken in various member states.

The newer version ISO 6579-1:2017 provides guidance of primary sampling methods and the preparation of samples before isolation, but also strain identification for a detailed epidemiological investigation. *Salmonella* spp. are isolated after pre-enrichment in buffered peptone water (BPW), resuscitating sub-lethally damaged or slow-growing *Salmonella* cells, thus minimizing false negative results. Pre-enrichment, also, helps the pathogen survive the toxic effect of selective enrichment media [14,15] when is present in very low numbers, as is the case with asymptomatic animals. However, pre-enrichment may not help isolation from faeces of the less vigorous *Salmonella* strains, such as the host-adapted ones. The problem in this case is the overgrowth of competing bacteria species multiplying faster than slow growing *Salmonella* during non-selective pre-enrichment [16]. When such a case is suspected, enrichment on modified semi-solid Rappaport–Vassiliadis (MSRV) medium supplemented with novobiocin is recommended. MSRV medium supplemented with novobiocin inhibits the growth of most Gram-positive and negative bacteria, helping the growth of less vigorous *Salmonella* spp. The introduction of this medium improved recovery as compared to previously used selective enrichment broths, such as Rappaport-Vassiliadis (soya base) (RVS) broth [17]. In addition, direct visualization of cultures minimizes time and material for confirming a definite negative result [18–21]. In all other cases, pre-enrinchment is followed by enrichment using selective plating on xylose-lysine-deoxycholate (XLD) agar, supplemented by an additional agar medium of choice enhancing the differentiation of salmonellae from other enterobacteria. This media combination, inhibiting growth of bacteria other than *Salmonella*, gives also important information on some principal differential biochemical characteristics, such as non-lactose fermentation and hydrogen sulphide (H_2S) production [22]. Exceptions are *Proteus*, *Pseudomonas*, *Citrobacter* and *Hafnia* [23,24] resembling *Salmonella* spp., with the exception of *S. arizonae* and *S. diarizonae*, fermenting lactose or variably producing H_2S. False negative cultures may also occur, if the low number of *Salmonella* present in a sample is suppressed by the overgrowth of other bacteria. Thus, quality of a sample, culture medium selectivity and also the incubation conditions affecting, perhaps, the expression of certain biochemical characteristics, influence prevalence.

For minimizing workload and false results, many chromogenic agars have been developed aiding the differentiation of suspect colonies [22,25,26]. Generally, chromogenic agars contain a combination of chromogenic substrates metabolized only by the targeted bacteria species, which produce colonies of distinctive coloring [27]. They are, however, serovar-dependent, thus frequently leading to false results [28] and also, relatively expensive, increasing the costs of routine laboratory procedures for animal testing [29]. In addition, the developing of culture media promising increased sensitivity and specificity is increasing inter-laboratory inconsistency, if standard procedures are not applied [30].

The various serological, biochemical and molecular tests applied require 5-11 days for a definitive confirmation of a suspect isolate [8]. *Salmonella* spp. are generally considered as non-lactose fermenting (NLFs), Gram negative rods, motile due to peritrichous flagellae (excluding serovars Pullorum and Gallinarum), fermenting glucose with the production of acid and usually gas, positive to catalase, methyl red and Simmons citrate reaction and negative to urease, lysine decarboxylase, indole, oxidase and Voges Proskauer (VP) tests. They produce H_2S and ferment L-arabinose, maltose, D-mannitol, D-mannose, L-rhamnose, D-sorbitol (except ssp VI), trehalose, D-xylose and dulcitol. These tests are now available in commercial identification kits, such as the API 20E (BioMιrieux) or the MicrogenTM GnA+B-ID (Microgen Bioproducts Ltd, UK) system. The small amounts of media used in these systems reduce the costs of and workload. Once a strain is identified as possibly belonging to *Salmonella* spp., it requires confirmation by serological testing for the detection of somatic (O), flagellar (H) and virulence (Vi) antigens possessed by salmonellae. However, accurate identification of a serovar is possible when the Kauffman–White Scheme is performed in a

 doi:10.20944/preprints202312.0762.v1

Salmonellae reference laboratory, where in case of doubtful results, phage typing and a genetic profile can be also obtained by experienced microbiologists, using the most appropriate culture methodology and media.

In consideration of the above, the objective of the present reporting is the dissemination of knowledge acquired from the observations and diagnostic difficulties resolved using the culture methods recommended by ISO 6579-1:2017 for the isolation of *Salmonella* spp. from swine samples collected at slaughter.

2. Materials and Methods

Samples, isolation and serotyping of Salmonella spp.

The observations reported refer to the testing of 615 tissue samples collected at slaughter from 123 randomly selected healthy pigs of 15 farrow-to- finish herds in Central Greece. Samples were collected after evisceration from the colon, ileum, mesenteric lymph nodes, gall-bladder and muscle tissue from the pigs' neck.

Isolation and typing procedures followed the Salmonella ISO 6579-1:2017 for food and animal feeding stuffs, as described elsewhere [31]. Suspect colonies were subcultured on Columbia blood agar (Oxoid, England), examined with Gram stain and if they were Gram negative rods, they were tested for oxidase production, utilization of Triple Sugar Iron Agar (Merck- Germany) and colony appearance on Salmonella Chromogenic Agar (SCA-Biolife-Italy). *Salmonella* suspect colonies showed on the SCA a magenta coloring after 18 to 24 h of incubation, while those of other members of the family Enterobacteriaceae appeared as blue or uncolored colonies. Suspect colonies were assigned to species using the API 20E (Biomerieux, France) and the MicrogenTM GnA+B-ID (Microgen Bioproducts Ltd, UK) Systems recommended for Gram (-) bacteria. Isolates identified as *Salmonella* spp. were tested with a polyvalent slide agglutination test (Remel Europe Ltd; Dartford, England) detecting O- and H- antigens and if positive, they were sent to the Greek National Reference Laboratory (GNRL) for specific serotyping. All lactose positive and H_2S negative isolates identified as *Salmonella* spp. were sub-cultured on Salmonella Shigella (SS) agar side by side with lactose negative- H_2S positive isolates.

Additionally, the swab sample and a loopful of BPW culture from the same sample were directly inoculated on XLD medium, by passing the pre-enrichment and enrichment stages.

3. Results

The GNRL recognized isolated *Salmonella* spp. as belonging to fourteen serovars. They were *S.* Typhimurium (21 isolates), *S. enterica* subsp. *enterica* ser. 4,12:i:- (9 isolates),S. *enterica* subsp. *enterica*. 6,7:k:- (7 isolates), *S. enterica* subsp. *enterica* ser. 4,5, 12:i:- (6 isolates), S. Bredeney (3 isolates) and one each S. Agona, S. Derby, S. Infantis, S. Meleagridis, S. Cerro, *S. enterica* subsp. *enterica* ser. 6,14,25: - : 1,2, S. *enterica* subsp. *diarizonae* 61:k:1,5, *S. enterica* subsp. *salamae* 38:b:1,2 and *S. enterica* subsp. *houtenae* 40:g,t:-. Four isolates were reported as 'Rough strains'.

Except two isolates, S. *enterica* subsp. *enterica* ser. 4,5, 12:i:- and S. *enterica* subsp. *enterica* ser. 6,14,25: - : 1,2, which had been isolated without enrichment (directly cultured from pre-enrichment on XLD), all others were isolated after enrichment.

3.1. Phenotypic expression of Salmonella isolates on selective media

The phenotypic expressions of *Salmonella* colonies on selected culture media are presented in Table 1. The accepted colony appearances for the various media used after incubation for 18 to 48 h are, magenta colonies on SCA, transparent colonies with or without black centers on SS, transparent colonies with or without black centers on XLD and transparent colonies on BGA.

Table 1. Atypical *Salmonella* isolates.

A/A	ISOLATE	MSRV	XLD	SS	BG	SCA	H_2S	TSI	ANTISERA	BIOCHEMICAL PROFILE
Typical colonial appearance		whitish opaque turbid zones with a diameter≥30 mm.	Red colonies with or without black centres	Straw coloured colonies with black centres	Red-pink-white opaque colonies	Magenta colonies.				
1.	S.I.4,5,12:i:-	-	b	a	a	a	+	a	*	*Salmonella* spp.
2.	S.I.4,5,12:i:-	-	a	a	a	a	+	a	*	*Salmonella* spp.
3.	S. I. 4,12:i:-	†††	b	a	a	a	+	a	*	*Salmonella* spp.
4.	S. I. 4,12:i:	††††	b	a	a	a	+	h	*	*Salmonella* spp.
5.	S. I. 4,12:i:	††††	b	a	a	a	+	a	*	*Salmonella* spp.
6.	S.I.6,14,25:-:1,2	-	c	d	e	f	+	g	*	*Salmonella* spp.
7.	*S. enterica* subsp. *houtenae* 40:g,t:-	†	c	d	e	f	+	g	*	*Salmonella* spp.
8.	*S. enterica* subsp. *salamae* 38:b:1,2	†	c	d	e	f	+	g	*	*Salmonella* spp.
9.	S.I. 6,7:k:-	†	c	d	e	a	+	g	*	*E.coli*
10.	S.I. 6,7:k:-	†††	c	d	e	a	+	g	*	*Salmonella* spp.
11.	*S*.Cerro	††	a	a	a	a	+	h	*	*E. coli* inactive
12.	*S. enterica* subsp. *diarizonae* 61:k:1,5	†††	a	a	a	a	+	h	*	

[a]Typical*Salmonella* colonies for the medium tested. [b] Pink transparent colonies with large skirt and a very small black center like a 'pinhead'. [c]Transparent colonies surrounded by yellow halos without black centers. [d]Red colonies (lactose positive), without black centers.When co-cultured with typical *Salmonella* isolates, they progressively (in 30 hours) exhibited the capability of producing H_2S, as well as they appeared as yellow colonies (lactose negative), giving thus the appearance of typical *Salmonella* spp. [e]Yellow to greenish yellow colonies and media, converted to red coloured colonies- red medium when co-cultured with typical *Salmonella* isolates. [f]Transparent colonies. [g] Yellow butt/slant and H_2S for the first 24h, but in 48h the butt changed to red. [h] Yellow butt/slant and very little H_2S production. [††] intermediate growth. [¶]slight positive reaction for both O,H antigens. † very little growth. †† intermediate growth. † †† very good growth. ††††excellent growth, the media turned to white. *Positive reaction for O,H antigens with polyvalent antisera. S.I: *S. enterica* subsp.*enterica.*

doi:10.20944/preprints202312.0762.v1

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 12 December 2023 doi:10.20944/preprints202312.0762.v1

Fifty six (94.92%) of the 59 Salmonella isolates transferred from MSRV to SCA medium and incubated for 24 hours produced magenta colonies and forty seven (79,66%) appeared as characteristic black colonies on XLD, SS and BG media due to production of H2S. S.enterica subsp. enterica ser. 6,14,25: - : 1,2, S. enterica subsp. houtenae 40:g,t:- and *S. enterica* subsp. *salamae* 38:b:1,2 appeared as transparent colonies on **SCA** medium (Figure 2) and as transparent colonies surrounded by yellow halos on **XLD** medium, without black centers. The same serovars appeared on **SS** agar as red colonies (lactose positive), without black centers. Interestingly, when they were co-cultured with Salmonella isolates of typical appearance on SS media, they progressively (in 30 hours) exhibited characteristics of H_2S production and appeared as yellow colonies (lactose negative), giving a delayed expression of typical *Salmonella* spp. (Figures 3 and 4). The above isolate produced yellow to greenish yellow colonies on **BG** agar, converted to red coloured colonies- red medium when co-cultured with *Salmonella* isolates of typical colony coloring. **TSI** test showed a delayed reaction giving a yellow butt/slant and H_2S production in 24h changing to a typical red after 48h of incubation. The above rare isolates showed very poor growth on **MSRV** compared to typical salmonellae. However, they agglutinated with the polyvalent antisera and their biochemical profiles (micro kits) were '*Salmonella* spp'. The above phenotypical profile was the same for two *S. enterica* subsp. *enterica*. 6,7:k:- isolates, which developed the characteristic agenta colonies on SCA, although one had been identified by both the API and Microgen systems as '*E. coli*'.

Three strains of *S. enterica* subsp. *enterica* ser. 4,12:i:- serovar and one of *S. enterica* subsp. *enterica* ser. 4,5,12:i:-, produced characteristic *Salmonella* colonies on SCA, SS and BG agars, but they appeared as pink transparent colonies with a large skirt and a very small black center like a 'pinhead' on XLD. The first three had a typical reaction when tested on TSI medium, while the last gave yellow butt/slant and very little H_2S production, showing also a weak growth on MSRV, as compared to the other three.

S. Cerro and *S. enterica* subsp. *diarizonae* 61:k:1,5 produced characteristic colonies on SCA and BG, but on XLD they produced transparent colonies without black centers and a red media. They also produced lactose negative (yellow) colonies with or without black centers on SS agar. S. Cerro gave on TSI agar yellow butt/slant and very little H_2S production. Interestingly, its biochemical profile given by MicrogenTMGnA+B-ID was 'inactive *E. coli*'. *S. enterica* subsp. *diarizonae* 61:k:1,5 gave red butt/yellow slant and very little H_2S production on TSI. Both isolates showed weak growth on MSRV, but a strong agglutination with the polyvalent antisera.

Finally, of the 615 samples cultured on MSRV 59 had no growth, 47 (7.64%) showed very strong growth (inoculated plates turned to white due to swarming) indicative of *Salmonella* spp. and 509 samples (82.76%) produced only zones of turbidity (Figure 1).

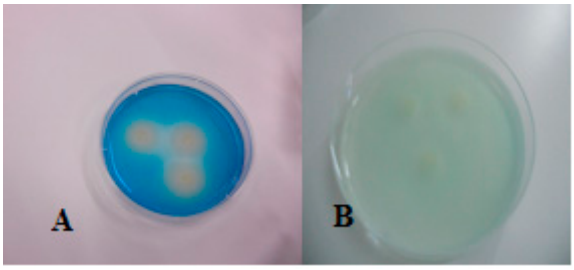

Figure 1. A: *E. coli* on MSRV, the medium does not inhibit the growth of the microorganism. **B:** *Salmonella* spp. on MSRV, the medium has a whitish colour.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 12 December 2023 doi:10.20944/preprints202312.0762.v1

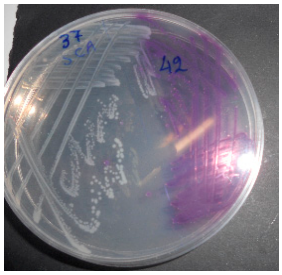

Figure 2. Transparent colonies of *S. enterica* subsp. *houtenae* 40:g,t:- (**left**), typical magenta colonies of *S.* Typhimurium (**right**) on SCA.

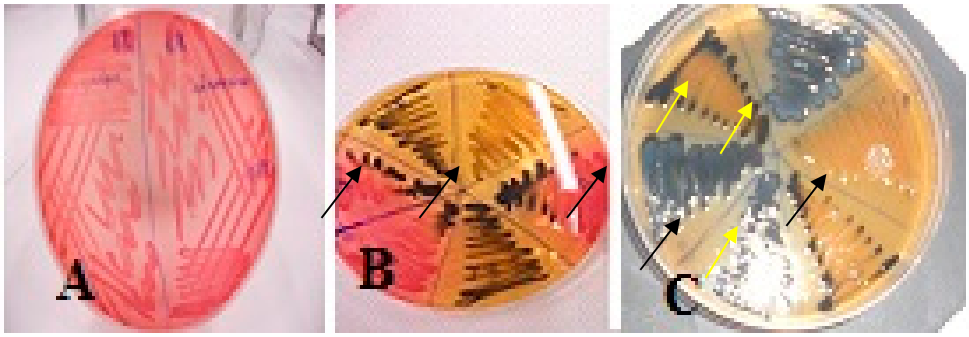

Figure 3. A: Lactose positive-H_2S(-) *Salmonella* isolates on SS agar after 24h. **B, C:** Lactose positive *Salmonella* isolates (black arrows) co-cultured with typical *Salmonella* isolates (yellow arrows) after 30h and after 48h respectively.

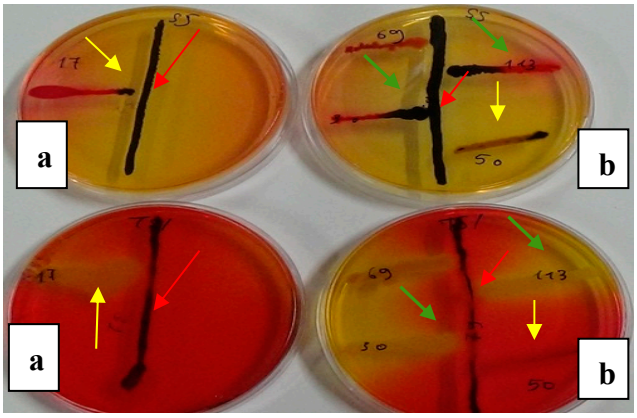

Figure 4. Typical *Salmonella* isolate (red arrows) on SS (a) and TSI (b) media co-cultured with: i) *Salmonella* H_2S (-)(strains 17, 50/ yellow arrows) and ii) *E.coli* (strains 30,113 /green arrows) after 36h incubation.

3.2. Phenotypic appearance of Gram-negative isolates other than Salmonella spp. on salmonella selective media

ISO 6579:2017 permitted the growth of eighteen Gram-negative isolates that resembled *Salmonella* spp. (transparent and/or black colonies on the selective media), eventually identified as E. coli (12 isolates), *Citrobacter freundii* (3 isolates), *Trabulsiella guamensis* (2 isolates) and *Klebsiella ozanae* (1 isolate). They all showed little to very good growth on MSRV agar (Table 2).

 doi:10.20944/preprints202312.0762.v1

Table 2. Enterobacteriaceae resembling *Salmonella*. Color of Colony and Media Utilization.

A/A	ISOLATE	MSRV	XLD	SS	BG	SCA	H_2S	TSI	*Salmonella* ANTISERA
8.	*E.coli*	-	a	a	a	f	+	p	O(+),H(-)
21.	*E.coli*	††	m	r	a	f	-	s	light (+) for both O,H antigens
30.	*E.coli*	†	x	d	o	f	-	s	O(+), H(+)
48.	*E.coli*	††	a	a	a	f	+	p	O(+),H(-)
52.	*E.coli*	†††	a	a	a	f	+	a	O(+), H(+)
76.	*E.coli*	†††	b	r	a	f	+	p	O(+),H(-)
77.	*E.coli*	†††	a	a	a	f	+	p	O(+),H(-)
80.	*E.coli*	††††	a	a	a	f	+	p	light (+) for both O,H antigens
81.	*E.coli* inactive	††	a	a	a	f	+	p	light (+) for both O,H antigens
82.		††	b	a	a	f	+	p	light (+) for both O,H antigens
88.	*E.coli* (indole negative)	††	y	t	o	f	+	p	light (+) for both O,H antigens
94.	*E.coli*	†	w	t	o	f	+	p	O(+),H(-)
75.	*Citrobacterfreundii* (indole negative)	†††	y	d	o	f	+	p	light (+) for both O,H antigens
83.	*Citrobacterfreundii*	†††	b	d	e	f	+	p	light (+) for both O,H antigens
93.	*Citrobacterfreundii*	†††	b	a	a	f	+	p	light (+) for both O,H antigens
4.	*Trabulsiellaguamensis*	††	k	a	o	f	+	p	light (+) for both O,H antigens
90.	*Trabulsiellaguamensis*	††	y	t	o	f	+	p	O (light +), H(-)
73.	*Klebsiellaozanae*	††	z	a	o	f	+	p	O,H (-)

[a]Typical *Salmonella* colonies for the medium tested. [f] Transparent colonies. [m]Transparent pink colonies without black centers/ red media. [p]Yellowbutt/ red slant, H_2S (+). [r] Lactose negative colonies without black centers. [s]Yellowbutt/ red slant, H_2S (-). [b] Pink transparent colonies with large skirt and a very small black center like a 'pinhead'. [d]Red colonies (lactose positive), without black centers.When co-cultured with typical *Salmonella* isolates, they progressively (in 30 hours) exhibited the capability of producing H_2S, as well as they appeared as yellow colonies (lactose negative), giving thus the appearance of typical *Salmonella* spp. [w]Large black colonies surrounded by reddish halos with yellow medium. [x]Yellow colonies(24h) converted to red coloniess(30h). [y]Yellow medium/ colonies with black centres. [z]Yellow medium/ colonies. [t]Lactose positive colonies with black centres. [o]Yellow to greenish yellow colonies and media.

Specifically, *E. coli* isolates produced pink transparent colonies with or without 'pinhead' black centers on XLD and transparent colorless colonies on SCA. Ten of them produced yellowish colonies on BG and red (lactose positive) colonies on SS. When they were co-cultured with typical *Salmonella* spp. isolates, they showed evidence of H_2S production and changed to yellow colonies (lactose negative) after 48h of incubation. The remaining two produced lactose negative colonies on SS agar with small black centers and transparent colonies with red media on BG. Ten of the twelve showed yellow butt/red slant on TSI agar and eight (8) produced H_2S. The remaining two produced yellow butt/yellow slant/H_2S(+) (Figure 4). Interestingly, seven of them agglutinated partly or totally with the polyvalent Salmonella antisera.

Two of the *C. freundii* isolates produced pink transparent colonies with black centers on XLD, while the third produced yellow colonies with black centers. Two of them produced lactose positive (red) colonies on SS agar and one lactose negative with black center. All of them showed colorless

 doi:10.20944/preprints202312.0762.v1

colonies on SCA and yellowish on BG agar. They had yellow butt/red slant/H_2S(+) on TSI agar and gave slight agglutination with the polyvalent Salmonella antisera. Two of them were indole negative.

One of the T.guamensis isolates resembled *Salmonella* on all the media evaluated, gave light agglutination with the polyvalent Salmonella antisera, but it was indole positive. The other produced yellow colonies with black centers on XLD. It did not agglutinate with the polyvalent O antisera, but it gave a very light positive reaction when tested with the polyvalent H antisera.

K. ozanae exhibited yellow colonies with small black centers on XLD, blue colonies on SCA, lactose negative colonies with large black centers on SS agar, transparent colonies on BG and gave yellow butt/slant and a 'trace' of H_2S production on TSI.

4. Discussion

A wide range of methodologies is available for the bacteriological isolation and characterization of *Salmonella* demanding the harmonization of results across the EU member states for *Salmonella* surveys and enforcement of regulations. This harmonization was enforced by the mandatory use of ISO 6579-1:2017. However, our observations show that the efficacy of the ISO needs further validation. Such problems of existing bacteriological methods and the need for faster reliable results, promote research for the development of molecular methods. However, for faecal and environmental samples deriving from pigs molecular methods are still in the early stages of development for giving consistent results. For now, the adoption of new methods in routine laboratory monitoring control programs is a compromise between improved sensitivity, speed, ease of use and economic benefits. However, compromise may result in the loss of some 'exotic' *Salmonella* serovars, negatively affecting their prevalence and eventually the effectiveness of control programmes.

Thus, studies held periodically to determine the sensitivity of the various new culture media becoming available show that some perform better than others. Their performance is, perhaps, influenced by the kind of sample (e.g. food, feed, faeces, environmental sources) or the species of the pathogen [32] infecting swine, with no culture method to be safely reliable in recovering all serovars [33–40]. Hence, recovery of *Salmonella* spp. from swine samples can be significantly different between culturing methods, with MSRV used for enrichment of swine feaces improving recovery, because it inhibits growth of most feacal bacteria species increasing in this way the sensitivity of XLD and BGA agars [12,20,36]. Their sensitivity after the use of MSRV is increased to 96% (in naturally contaminated food samples) compared to the standard methods [41].

In the present work 47 (7.64%) of 615 samples cultured on MSRV showed extensive swarming indicative of *Salmonella's* migration through the MSRV medium and expressed as turbidity (whitening of the medium). Unfortunately, of the 509 samples producing minimal turbidity, nine (9) were eventually identified as *Salmonella*, a number reducing the specificity of MSRV for *Salmonella* spp., which proved to be very poor for this specific medium (81.3% false positive results on the factor of inhibiting growth of non-*Salmonella* species). The reduction of specificity necessitates complementary testing increasing labor and costs, in order to differentiate salmonellae from other microorganisms. Perhaps, pre-enrichment needs optimization according to a sample's suspect microbial burden, helping in this manner also the recovery of damaged or slow-growing *Salmonella*.

Of interest was also the direct recovery of three *Salmonella* isolates from non-selective pre-enrichment broth. These isolates had been lost on enrichment media (MSRV) as their inability to grow on this medium was confirmed. MSRV medium had, perhaps, a killing effect on this isolates affecting in this manner the prevalence of *Salmonella* spp. in an animal population or food stuff. The finding implies that maximum detection can result from the combination of selective enrichment and direct plating from pre-enrichment media. This is however, unacceptable for routine testing, when large numbers of samples are tested.

Evident was also, that the choice of differential plating culture media significantly affects recovery. The study confirmed that most, but not all *Salmonella* serovars grow adequately on XLD, SS, BG and SCA. With the exception of the three rare *Salmonella* serovars, SCA appeared as a medium with better specificity than the combination of XLD, SS, BGA and TSI. Similar problems of non-specific and difficult to interpret results recorded here during the use of the former version, ISO

6579:2002/Amd 1:2007 (Annex D) have been previously reported [22] and show that false positives (e.g. *Citrobacter*, *Proteus*), increase the workload and costs of a control program.

A previous comparison between XLD and BGA media showed that BGA is inferior to XLD, due to its higher selectivity [42,43] and the same was observed in the present study. Although use of chromogenic media makes recognition of most salmonellae easier, the present study showed that they fail in cases of rarer *Salmonella* serovars. These serovars could become of epidemiological importance in later times, if they are not accounted during control programs. Thus, failure of culture methods to recognize some *Salmonella* serovars, lead to a false assessment of the infectiousness of a given sample [40]. The suppression of competing bacteria in the original sample to allow the growth of *Salmonella* to the level of detection is important, but it may also affect sensitivity of the conventional culturing media used. Sensitivity could be improved with the use of multiple culturing methods in parallel, but the combination increases workload and costs.

Of interest was also the detection of lactose positive *Salmonella* isolates, resembling *E. coli* on XLD and SS agars. Lactose fermentation is a biochemical test differentiating *Salmonella* from other Enterobacteriaceae. *Escherichia coli* is since 1887, recognized as a lactose fermenter and *Salmonella* as a non-lactose fermenter. Therefore, this information was used for the development of the differential agar media, XLD, SS and BG. However, a few decades ago Ewing (1986) [44] reported that about 1% of all salmonellae ferment lactose, but the true incidence of lactose-fermenting (lac+) salmonellae is yet unknown, because most diagnostic laboratories do not further characterized lactose positive colonies [45]. Lac+*Salmonella* strains of some important serovars, such as serovar Typhimurium, have been reported [46–48] from animal populations and it is important to be accounted during the application of control programs or during the investigation of suspect food-borne human cases.

The types of colonies examined here, would not have been selected for further testing, if the ISO was strictly followed, because they resembled typical lac+ *E. coli.* This would have resulted in their underreporting. False negative samples may also occur, if *Salmonella* lac+ strains are masked by growth of bacteria, such as *Klebsiella* spp., carrying the lactose (lac) operon on a plasmid or a chromosome [49,50].

Another important taxonomic characteristic of Enterobacteriaceae is the production of H_2S. *Citrobacter*, *Proteus* and *Salmonella* are major H_2S-producing genera. H_2S production is, perhaps, helping the colonization of gut and in synergy with other virulence determinants, the development of gastroenteritis [51]. About 97% of *Salmonella* spp., with the exception of *S*. Choleraesuis and *S*. Typhi [52], produce H_2S. However, this characteristic may be absent in environmental strains of *Salmonella* spp. due to mutations [53,54]. One pathway of H_2S production is through the reduction of thiosulfate mediated by the enzymethiosulfate reductase [51,55,56]. However, there are some problems in the interpretation of H_2S production needing further consideration. Specifically,

i) the content and availability of the sulphur source and peptones of the culture media. Peptones themselves contain varying amounts of sulfur amino acids and partially oxidized sulfur compounds, giving potentially a false positive result,

ii) the commercial diagnostic media may also contain fermentable carbohydrates, resulting in the masking of H_2S production by the production of acid during sugar fermentation either because it is repressed or because it cannot be detected,

iii) the ability of the microorganism to stimulate H2S-producing enzymes and its H_2S-producing rate [51,55,57]. Some consider that the ability of an isolate to produce H_2S is plasmid mediated [58–62], although the nature of the plasmid differs from isolate to isolate. The above could be the reason that lactose-positive *Salmonella* spp. appeared H_2S (-) on XLD and SS agars, possibly influenced by fermentation [63]. In the present study non H_2S producing *Salmonella* and *E. coli* isolates were stimulated to produce it when cultured side by side with H_2S producing isolates. Perhaps, the 'masking' of H_2S was overcome in synergy when lactose negative and strong H_2S-producing isolates were cultured side by side. However, false negative results decrease the recovery of *Salmonella* spp., if ISO recommendations are strictly followed, influencing its diagnostic value.

Of similar diagnostic importance is the isolation of *E. coli* strains producing H_2S and resembling *Salmonella* on XLD. The inability of *E. coli* to produce detectable amounts of H_2S on recommended

culture media is a differential characteristic of the genus *Escherichia*, although H_2S -producing variants have been isolated on numerous occasions [59,60,62,64–66]. Researchers suggest that the ability of *E. coli* to produce H_2S is transferable between microorganisms in a similar way with transfer of hemolysins, colicins, plasmids for drug resistance and enterotoxin [60]. Although the isolation of H_2S-producing variants of *E. coli* is infrequent [59] and their clinical significance remains unclear, their presence in specimens influence the diagnostic process due to misclassifications. As to the role of H_2S-producing microorganisms in the gut, one could think that they may synergistically reactivate the 'masked' ability of H_2S producing *Salmonella* spp. and the abundant in the gut *E. coli* increasing their virulence.

Additionally, such diagnostic problems could negatively affect national schemes of controlling *Salmonella* infections in animals in order to protect the consumer. Surveillance of *Salmonella* in all stages of the food chain is important in the epidemiology of food-borne salmonellosis, thus, the development and successful implementation of control strategies. Their success is largely dependent on reliable laboratory methods for the isolation, identification and typing of suspect colonies. Diagnostic laboratories must choose isolation and identification approaches that provide efficiently and accurately results comparable between laboratories. The development of standard culture methods, as ISO 6579-1:2017, and participation in proficiency quality assurance programs helps harmonization between laboratories. However, there isn't a single method fulfilling all the criteria or being optimal for all purposes and in some cases complementary biochemical tests are necessary. Recognizing the limits of the currently used selective and differential media is helping the improvement of their specificity and sensitivity, thus the differentiation of *Salmonella* spp. from other Enterobacteriaceae, sharing common biochemical and antigenic characteristics, but remaining cost-effective. Reliability, ease of use and costs are characterizing conventional selective and differential media as compared to the newer chromogenic ones not yet standing in time. The use of two conventional selective media with different selective and differentiating properties in conjunction with experience of the bench technician are likely to help in the detection of atypical strains, like the lactose fermenting, which in most cases are discarded as not important. The objective should always be to maximize detection of major zoonotic *Salmonella* strains [67]. Thus, the working protocol should aim toward this objective, which will be further achieved if the microbiologist is scientifically prepared to detect those considered "rarer". These rarer ones, perhaps, of little epidemiological importance today, could become a potential threat in the future. Failure to recognize atypical strains of *Salmonella* spp. could result in considerable epidemiological problems in later years.

Author Contributions: Conceptualization, methodology, investigation, writing—original draft preparation, Grammato Evangelopoulou; Angeliki R Burriel; software, resources, supervision, Nikolaos Solomakos. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Majowicz SE, Musto J, Scallan E, Angulo FJ, Kirk M, O'Brien SJ, Jones TF, Fazil A, Hoekstra RM. The global burden of nontyphoidal *Salmonella* gastroenteritis.*Clin. Infect. Dis.* **2010**, *50*, 882–889.
2. EFSA and ECDC (European Food Safety Authority and European Centre for Disease Prevention and Control), 2022. The European Union One Health 2021 Zoonoses Report. *EFSA J.* **2022**, 20(12):7666, 273 pp. https://doi.org/10.2903/j.efsa.2022.7666
3. Pires SM, de Knegt L, Hald T. Estimation of the relative contribution of different food and animal sources to human *Salmonella* infections in the European Union.Scientific/technical report.European Food SafetyAuthority, Parma, Italy, 2011.
4. Chaname Pinedo L, Mughini-Gras L, Franz E, Hald T and Pires SM. Sources and trends of human salmonellosis in Europe, 2015–2019: an analysis of outbreak data. *Int. J. Food Microbiol.* **2022**, 379, 109850. https://doi.org/10.1016/j.ijfoodmicro.2022.109850
5. Alocilja EC, Radke SM. Market analysis of biosensors for food safety. *Biosens. Bioelectron*, **2003**, 18(5-6):841-846. doi.org/10.1016/S0956-5663(03)00009-5

6. Hurd HS, McKean JD, Griffith RD, Rostagno MH. Estimation of the *Salmonella enterica* prevalence in finishing swine. *Epidemiol Infect*. **2004**, 132, 127–135.
7. Waltman WD, Mallinson ET. Isolation of *Salmonella* from poultry tissue and environmental samples: a nationwide survey. *Avian Dis.* **1995**, 39, 45–54.
8. ISO (International Organization for Standardization). ISO 6579-1:2017. Microbiology of the food chain -- Horizontal method for the detection, enumeration and serotyping of Salmonella -- Part 1: Detection of Salmonella spp. ISO, Geneva, Switzerland, **2017**.
9. European Food Safety Authority. Report of the Task Force on Zoonoses Data Collection on the analysis of the baseline survey on the prevalence of *Salmonella* in slaughter pigs, in the EU, 2006-2007. Part A. Salmonella prevalence estimates. *EFSA J*. **2008**, 135, 1–111.
10. European Food Safety Authority. Analysis of the baseline survey onthe prevalence of *Salmonella* in holdings with breeding pigs in the EU, 2008. Part A. *Salmonella* prevalence estimates. *EFSA J.* **2009**, 7:1–93.
11. EFSA (European Food Safety Authority), Boelaert F, Papanikolaou A, Rizzi V and Stoicescu A-V, 2020. Manual for reporting on zoonoses and zoonotic agents, within the framework of Directive 2003/99/EC, and on some other pathogenic microbiological agents for information derived from the year 2019. EFSA supporting publication **2020**: EN-1791. 63 pp. https://doi.org/10.2903/sp.efsa.2020.EN1791
12. Eriksson E, Aspan A. Comparison of culture, ELISA and PCR techniques for *Salmonella* detection in faecal samples for cattle, pig and poultry. *BMC Vet. Res.* **2007**, 3:21.
13. ISO (International Organization for Standardization). ISO 6579:2002/Amd 1:2007. Detection of *Salmonella* spp. in animal faeces and in environmental samples from the primary production stage, Amendment 1, Annex D. In Microbiology of food and animal feeding stuffs. Horizontal method for the detection of *Salmonella* spp. International Organization for Standardization, Geneva, Switzerland, **2007**.
14. Baylis CL, MacPhee S, Betts RP. Comparison of two commercial preparations of buffered peptone water for the recovery and growth of *Salmonella* bacteria from foods. *J. Appl. Microbiol*. **2000**, 89(3), 501-10.
15. Zheng Q, Mikˇs-Krajnik M, D'Souza C et al. Growth of healthy and sanitizer-injured *Salmonella* cells on mug bean sprouts in different commercial enrichment broths. *Food Microbiol*. **2015**, 52, 159–168,.
16. Gracias KS, McKillip JL. A review of conventional detection and enumeration methods for pathogenic bacteria in food. *Can. J. Microbiol*. **2004**, 50(11), 883-890.
17. Zdragas A, Tsakos P, Mavrogeni P. Evaluation of two assays, MSRV and RV, for the isolation of *Salmonella* spp. from wastewater samples and broiler chickens. *Lett. Appl. Microbiol*. **2000**, 31, 328-31.
18. Read SC, Irwin RJ, Poppe C, Harris J. A comparison of two methods for isolation of *Salmonella* from poultry litter samples. *Poult. Sci.* **1994**, 73, 1617-1621.
19. Davies RH, Wray C. Evaluation of a rapid cultural method for identification of salmonellas in naturally contaminated veterinary samples. *J. appl. Bacteriol*. **1994**, 77, 237- 241.
20. Voogt N, Raes M, Wannet WJ, Henken AM, Giessen AW van de. Comparison of selective enrichment media for the detection of *Salmonella* in poultry faeces. *Lett. Appl. Microbiol.* **2001**,32(2):89-92.
21. Dam-Deisz WD, Maas HM, Nagelkerke N, van de Giessen AW. Comparison of selective enrichment media for the isolation of *Salmonella* spp. from faecal samples from fattening pigs, veal calves and dairy cows. *De Ware(n) Chemicus*. , **2003**, 3, 143-151.
22. Manafi M. New developments in chromogenic and fluorogenic culture media. *Int. J. Food Microbiol.* **2000**. 60, 205-18.
23. Tate CR, Miller RG, Mallinson ET, Douglass LW, Johnston RW. The isolation of salmonellae from poultry environmental samples by several enrichment procedures using plating media with and without novobiocin. *Poult. Sci.* **1990**, 69:721–726.
24. Eigner U, Reissbrodt R, HammannR,Fahr AM. Evaluation of a new chromogenic medium for the isolation and presumptive identification of *Salmonella* species from stool specimens. *Eur. J. Clin. Microbiol.Infect. Dis.* **2001**, 20,558 –565.
25. Perry JD, Ford M, Taylor J, Jones AL, Freeman R, Gould FK. ABC medium, a new chromogenic agar for selective isolation of *Salmonella* spp. *J. Clin.Microbiol.* **1999**, 37, 766 –768.
26. Schönenbrücher V, MallinsonET,Bülte M. A comparison of standard cultural methods for the detection of foodborne Sa*lmonella* species including three new chromogenic plating media. *Int. J. Food Microbiol*. **2008**, 123:61– 66.
27. Ruiz J, Núñez ML, Lorente I, Pérez J, Simarro E, Gómez J. Performance of six culture media for isolation of *Salmonella* species from stool samples. *Eur. J. Clin. Microbiol. Infect. Dis.* **1996**, 15, 922–926.
28. Ruiz J, Nunez ML, Diaz J, Lorente I, Perez J, Gomez J. Comparison of five plating media for isolation of *Salmonella* species from human stools. *J. Clin. Microbiol.* **1996**, 34, 686–688.
29. Perry JD, Freydière AM. The application of chromogenic media in clinical microbiology. *Lett. Appl. Microbiol*. **2007**, 103, 2046 –2055.
30. Hyatt DR, Weese JS. *Salmonella* Culture: sampling procedures and laboratory techniques. *Vet. Clin. North Am. Equine Pract.* **2004**, 20(3), 577-85. 10.

31. Evangelopoulou G, Kritas S, Govaris A, Burriel AR. Pork Meat as a Potential Source of *Salmonella enterica* subsp. *arizonae* Infection of Man. *J. Clin. Microbiol*. **2014**, 52, 741-744. https://doi.org/10.1128/JCM.02933-13)
32. Love BC, RostagnoMH. Comparison of Five Culture Methods for *Salmonella* Isolation from Swine Fecal Samples of Known Infection Status. *J. VET. Diagn. Invest.* **2008** 20: 620. https://doi.org/10.1177/104063870802000514.
33. Davies PR, Turkson PK, Funk JA, Nichols MA, Ladely SR, Fedorka-Cray PJ. Comparison of methods for isolating *Salmonella* bacteria from faeces of naturally infected pigs. *J. Appl. Microbiol.* **2000**, 89, 169–177.
34. Jensen AN, Sørensen G, Baggesen DL, Bødker R, Hoorfar J. Addition of Novobiocin in pre-enrichment step can improve Salmonellaculture protocol of modified semisolid Rappaport-Vassiliadis. *J. Microbiol. Methods*. **2003**, 55:249–255.
35. Osumi T, Asai T, Namimatsu T, Sato S, Yamamoto K. Enrichment for isolating *Salmonella* choleraesuis and other *Salmonella* spp.from pigs. *J. Vet. Med. Sci*. **2003**, 65:949–951.
36. Pangloli P, Dje Y, Oliver SP, Mathew A, Golden DA, Taylor WJ, Draughon FA. Evaluation of methods for recovery of Salmonella from dairy cattle, poultry, and swine farms. Evaluation of methods for recovery of *Salmonella* from dairy cattle, poultry, and swine farms. *J. Food Prot.* **2003**, 66, 1987–1995.
37. Korsak N, Degeye JN, Etienne G, China B, Daube G. Comparison offour different methods for Salmonella detection in fecalsamples of porcine origin. *J. Food Prot*. **2004**, 67:2158–2164.
38. Champagne MJ, Ravel A, Daignault D. A comparison of sample weight and culture methods for the detection of *Salmonella* in pig feces. *J. Food Prot*. **2005**, 68, 1073–1076.
39. Mejia W, Zapata D, Mateu E, Martin M. Lack of specificity of a combination of Rappaport-Vassiliadis broth and XLT4 agar for the isolation of salmonellae from pig faeces. *Vet. Rec*. **2005**, 156, 150–151.
40. Rostagno MH, Gailey JK, Hurd HS, Mckean JD, Leite RC. Culture methods differ on the isolation of *Salmonella enterica* serotypes from naturally contaminated swine fecal samples. J. *Vet. Diagn. Invest*. **2005**, 17:80–83.
41. De Zutter L, De Smedt JM, Abrams R, Beckers H, Catteau M, de Borchgrave J,Debevere J, Hoekstra J, Jonkers F, Lenges J, Notermans S, Van Damme L, Vandermeersch R, Verbraeken R, Waes G. Collaborative study on the use of motility enrichment on modified semisolid Rappaport-Vassiliadis medium for the detection of *Salmonella* from foods. *Int. J. Food Microbiol*. **1991**, 13, 11–20.
42. Bauwens L, Vercammen F, Bertrand S, Collard JM, De Ceuster S. Isolation of *Salmonella* from environmental samples collected in the reptile department of Antwerp Zoo using different selective methods. *J. Appl. Microbiol. 2006*, 101(2):284-289.
43. Koyuncu S, Haggblom P. A comparative study of cultural methods for the detection of *Salmonella* in feed and feed ingredients. *BMC Vet. Res*. **2009**, 5:6. https://doi.org/10.1186/1746-6148-5-6.
44. Ewing WH. Differentiation of Enterobacteriaceae by biochemical reactions, p. 47–72. In Edwards and Ewing's identification of the Enterobacteriaceae,4th ed. Elsevier Science Publishing Co., Inc., New York, N.Y., 1986.
45. Falcao DP, Trabulsi LR, Hickman F, Farner JS. Unusual Enterobacteriaceae: lactosepositive *Salmonella* typhimurium which is endemicin Sao-Paulo, Brazil. *J. Clin. Microbiol*. **1975**, 2:349-353.
46. Rokey NW Mecca MD. Lactose fermenting salmonellae—a dilemma for diagnostic laboratories? *Proc. Annu. Meet. U.S. Anim. Health Assoc*. **1972**, 75, 509–514.
47. Blackburn BO Ellis EM.. Lactose-fermenting *Salmonella* from dried milk and milk-drying plants. *Appl. Microbiol.* **1973**, 26, 672–674.
48. Johnston KG, Jones RT. Salmonellosis in calves due to lactose fermenting *Salmonella* typhimurium. *Vet. Rec.* **1976**, 98,276–278.
49. MacDonald C, Riley M.. Cloning chromosomal lac genes of Klebsiella pneumoniae. *Gene*, **1983**, 24:341–345.
50. Patrick L. McDonough, Sang J. Shin and Donald H. Lein. Diagnostic and Public Health Dilemma of Lactose-Fermenting *Salmonella enterica* Serotype Typhimurium in Cattle in the Northeastern United States. *J.Clin.Microbiol*. **2000**, 38(3):1221.
51. Barrett EL, Clark MA. Tetrathionate Reduction and Production of Hydrogen Sulfide from Thiosulfate. *Microbiol. Rev*. **1987**, 51:192-205.
52. Ewing WH. Edwards and Ewing's identification of Enterobacteriaceae, 4th ed., p. 192. Elsevier, Amsterdam, 1985.
53. Lannigan R, Hussain Z. Wound Isolate of *Salmonella* typhimurium That Became Chlorate Resistant after Exposure to Dakin's Solution: Concomitant Loss of Hydrogen Sulfide Production, Gas Production, and Nitrate Reduction. *J. Clin.Microbiol*. **1993**, 31(9):2497.
54. Sasahara KC, Heinzinger NK, Barrett EL. Hydrogen Sulfide Production and Fermentative Gas Production by *Salmonella* yphimurium Require F0F1 ATP Synthase Activity. *J.Bacteriol*. **1997**, 179, 6736–6740.
55. Clarke PH. Hydrogen Sulphide Production by Bacteria. *J .gen. Microbiol*. **1953,** 8, 397407.
56. Leinweber FJ, Monty KJ. The metabolism of thiosulphate in *Salmonella* typhimurium. *J. Biol. Chem*. **1963**, 238, 3775-3780.

57. Rambach A. New plate medium for facilitated differentiation of *Salmonella* spp. from *Proteus* spp. and other enteric bacteria. *Appl. Environ.Microbiol*. **1990**, 56, 301–303.
58. Burkardt HJ, Mattes R, Schmid K, Schmitt R. Properties of two conjugative plasmids mediating tetracyclineresistance, raffinose catabolism, and hydrogen sulfide production by *Escherichia coli*. *Mol. Gen. Genet*. **1978**, 166, 75-84.
59. Lautrop H, Orskov I, Gaarslev K. Hydrogensulphide producing variants of *Escherichia coli. Acta Pathol. Microbiol. Scand*. **1971**, 79, 641-650.
60. Layne P, Hu ASL, Balows A, Davis BR. Extrachromosomal nature of hydrogen sulfide productionin Escherichia coli. *J. Bacteriol*. **1971**, 106, 1029-1030.
61. Magalhies M, Veras A.. Properties of pPE1572(Hys-Raf), a plasmid governing hydrogen sulphide production and raffinose fermentation in *Escherichia coli*. *J. Gen. Microbiol*. **1977**, 99, 445-447.
62. 0rskov I, 0rskov F. Plasmid-determined H_2S character in *Escherichia coli* and its relation to plasmid-carried raffinose fermentation and tetracycline resistance characters. Examination of 32 H_2S-positive strains isolated during theyears 1950-1971. *J. Gen. Microbiol*. **1973**, 77, 487-499.
63. Bulmash JM, Fulton M, Jiran J. Lactose and sulfide reactions of an aberrant *Salmonella* strain. J. *Bacteriol.* **1965**, 89:259.
64. Barbour EK, Nabbut NH, Al-Nakhli HM.. Production of H_2S by *Escherichia coli* isolated from poultry: an unusual character useful for epidemiology of colisepticemia. *Avian Dis*. **1985**, 29, 341-346.
65. Darland G, Davis BR.. Biochemical and serological characterization of hydrogen sulfide-positive variants of *Escherichia coli*. *Appl. Microbiol*. **1974**, 27, 54-58.
66. Maker MD, Washington JA II.. Hydrogensulfide-producing variants of *Escherichia coli. Appl. Microbiol*. **1974**, 28, 303-305.
67. Carrique-Mas JJ, Davies RH. Sampling and bacteriological detection of *Salmonella* in poultry and poultry premises: a review. *Rev. sci. tech. Off. int. Epiz*. **2008**, 27 (3), 665-677.