

## Article

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

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**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 protocol officially recommended by the EU for the isolation of *Salmonella* spp., aiming for the harmonization of effective control of *Salmonella* infection in food-producing animals. Successful control of animal salmonellosis is highly dependent on the sensitivity and reliability of the biochemical methods used to detect 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 other bacteria antigenically resembling salmonellae. An evaluation of the culture media was conducted, including 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 for the isolation of *Salmonella* from swine samples. It was concluded that the presence of atypical *Salmonella* strains negatively affects the prevalence of *Salmonella*, thus affecting the identification of carrier pigs and eventually affecting the efficiency of control programs. Thus, doubtful results require additional biochemical testing to confirm the accuracy of such universally recommended isolation methods.

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



**Citation:** Evangelopoulou, G.; Burriel, A.R.; Solomakos, N. Distinctive Culture Expressions of Enterobacteria Interfering with Isolation of *Salmonella* spp. during the Application of the Recommended ISO 6579-1:2017. *Appl. Sci.* **2024**, *14*, 953. <https://doi.org/10.3390/app14030953>

Academic Editor: Monica Gallo

Received: 7 December 2023

Revised: 11 January 2024

Accepted: 19 January 2024

Published: 23 January 2024



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## 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 infection with *Salmonella* spp. [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, which depends on the sampling plans, sample points (surfaces of the slaughterhouse or part of the pork tested for presence of *Salmonella*), and number of grams/surface area tested; thus, isolation of the agent [5,6] uses a combination of culture media. Some of them are, due to their selectivity and sensitivity, the ‘gold standard’ for *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], which has become 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 commercially available systems based on enzyme-linked immunosorbent 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 and thus the coordination of control programs undertaken in various member states.

The newer version, ISO 6579-1:2017, provides guidance for primary sampling methods and the preparation of samples before isolation, but also for 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 to survive the toxic effects of selective enrichment media [14,15] when it is present in very low numbers, as is the case with asymptomatic animals. However, pre-enrichment may not help with isolation from feces 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 with the growth of less vigorous *Salmonella* spp. The introduction of this medium improved recovery 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-enrichment is followed by enrichment using selective plating on xylose-lysine-deoxycholate (XLD) agar and is supplemented by an additional agar medium of choice, which enhances the differentiation of salmonellae from other enterobacteria. This media combination, which inhibits the growth of bacteria other than *Salmonella*, also gives important information on some principal differential biochemical characteristics, such as non-lactose fermentation and hydrogen sulfide (H<sub>2</sub>S) production [22]. Exceptions are *Proteus*, *Pseudomonas*, *Citrobacter*, and *Hafnia* [23,24] species resembling *Salmonella* spp., and also *S. arizonae* and *S. diarizonae*, that ferment lactose or variably produce H<sub>2</sub>S. False-negative cultures may also occur if the low number of *Salmonella* bacteria present in a sample is suppressed by the overgrowth of other bacteria. Thus, the quality of a sample, culture medium selectivity, and also the incubation conditions affecting, perhaps, the expression of certain biochemical characteristics, influence prevalence.

In order to minimize workloads and false results, many chromogenic agars have been developed to aid with 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 they are also relatively expensive, increasing the costs of routine laboratory procedures for animal testing [29]. In addition, the development of culture media promising increased sensitivity and specificity increases 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 to be non-lactose-fermenting (NLFs); Gram-negative rods; motile due to peritrichous flagellae (excluding serovars Pullorum and Gallinarum); glucose fermenting, 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<sub>2</sub>S 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 (BioMérieux) or the Microgen™ GnA+B-ID (Microgen Bioproducts Ltd., Camberley, Surrey, UK) systems. The small amounts of media used in these systems reduce both cost and workload. Once a strain is identified as possibly belonging to *Salmonella* spp., this 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 Salmonellae reference laboratory where, in the 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, Basingstoke, UK) and 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, Darmstadt, Germany), and colony appearance on Salmonella Chromogenic Agar (SCA-Biolife-Italy, Milan, Italy). *Salmonella*-suspect colonies showed magenta coloring on the SCA after 18 to 24 h of incubation, while colonies of other members of the family Enterobacteriaceae appeared as blue or uncolored. Suspect colonies were assigned to species using the API 20E (Biomérieux, Craponne, France) and the Microgen™ GnA+B-ID (Microgen Bioproducts Ltd., Camberley, UK) systems recommended for Gram (–) bacteria. Isolates identified as *Salmonella* spp. were tested with a polyvalent slide agglutination test (Remel Europe Ltd., Dartford, UK) detecting O- and H- antigens, and positive samples were sent to the Greek National Reference Laboratory (GNRL) for specific serotyping. All lactose-positive and H<sub>2</sub>S-negative isolates identified as *Salmonella* spp. were subcultured on Salmonella–Shigella (SS) agar side by side with lactose-negative-H<sub>2</sub>S-positive isolates.

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

## 3. Results

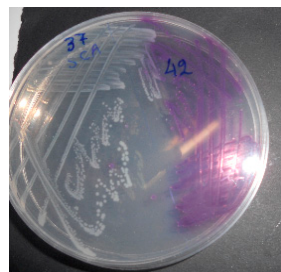
The GNRL recognized isolated *Salmonella* spp. as belonging to fourteen serovars. These were *S. Typhimurium* (21 isolates), *S. enterica* subsp. *enterica* ser. 4,12:i:- (9 isolates), *S. enterica* subsp. *enterica*. 6,7:k:- (7 isolates), *S. enterica* subsp. *enterica* ser. 4,5,12:i:- (6 isolates), *S. Bredeney* (3 isolates), and one each of *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 for 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.

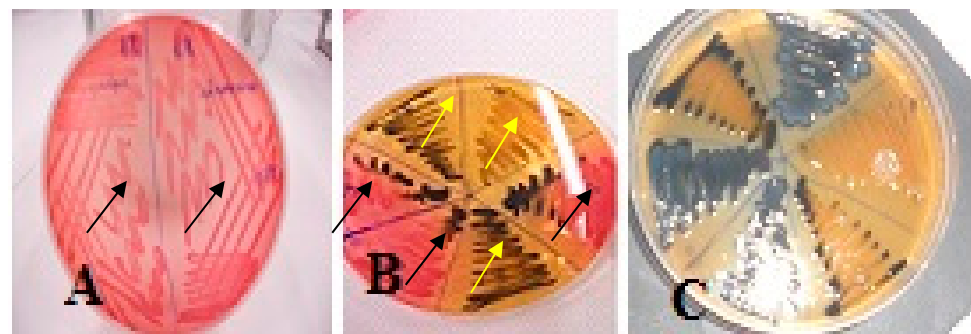
### 3.1. Phenotypic Expression of *Salmonella* Isolates on Selective Media

The phenotypic expression 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.

Fifty six (94.92%) of the 59 *Salmonella* isolates transferred from MSR/V to SCA medium and incubated for 24 h produced magenta colonies and forty seven (79.66%) appeared as characteristic black colonies on XLD, SS, and BG media due to production of H<sub>2</sub>S. *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 1) 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 h) exhibited characteristics of H<sub>2</sub>S production and appeared as yellow colonies (lactose-negative), giving a delayed expression of typical *Salmonella* spp. (Figures 2 and 3). The above isolate produced yellow to greenish-yellow colonies on BG agar, which converted to red-colored colonies and red medium when co-cultured with *Salmonella* isolates of typical colony coloring. TSI testing showed a delayed reaction giving a yellow butt/slant and H<sub>2</sub>S production in 24 h that changed to a typical red after 48 h of incubation. The above rare isolates showed very poor growth on MSR/V compared to typical salmonellae. However, they agglutinated with the polyvalent antisera and their biochemical profiles (micro kits) were '*Salmonella* spp.'. The phenotypical profile described above was the same for two *S. enterica* subsp. *enterica*. 6,7:k:- isolates, which developed the characteristic magenta colonies on SCA, although one had been identified by both the API and Microgen systems as '*E. coli*'.



**Figure 1.** Transparent colonies of *S. enterica* subsp. *houtenae* 40:g,t:- (left) and typical magenta colonies of *S. Typhimurium* (right) on SCA.



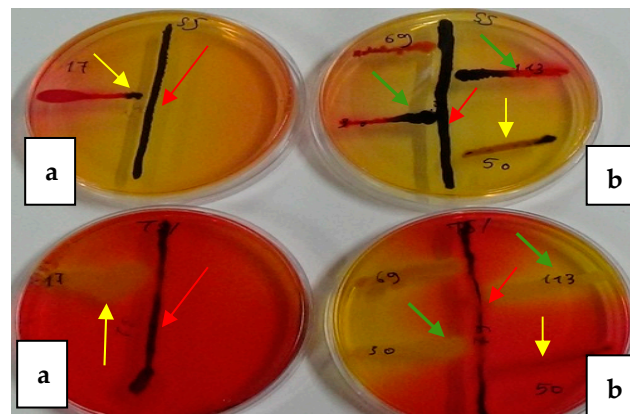
**Figure 2.** (A) Lactose-positive-H<sub>2</sub>S (-) *Salmonella* isolates on SS agar after 24 h. (B,C) Lactose-positive *Salmonella* isolates (black arrows) co-cultured with typical *Salmonella* isolates (yellow arrows) after 30 h and after 48 h, respectively.

**Table 1.** Atypical *Salmonella* isolates.

A/A		MSRV	XLD	SS	BG	SCA	H <sub>2</sub> S	TSI		
Typical Colonial Appearance	Isolate	Whitish-Opaque Turbid Zones with a Diameter ≥ 30 mm	Red Colonies with or without Black Centers	Straw-Colored Colonies with Black Centers	Red-Pink-White Opaque Colonies	Magenta Colonies			Antisera	Biochemical Profile
1.	S.I.4,5,12:i:-	–	b	a	a	a	+	a	*	<i>Salmonella</i> spp.
2.	S.I.4,5,12:i:-	–	a	a	a	a	+	a	*	<i>Salmonella</i> spp.
3.	S.I.4,12:i:-	+++	b	a	a	a	+	a	*	<i>Salmonella</i> spp.
4.	S.I.4,12:i:	++++	b	a	a	a	+	h	*	<i>Salmonella</i> spp.
5.	S.I.4,12:i:	++++	b	a	a	a	+	a	*	<i>Salmonella</i> spp.
6.	S.I.6,14,25:-:1,2	–	c	d	e	f	+	g	*	<i>Salmonella</i> spp.
7.	<i>S. enterica</i> subsp. <i>houtenae</i> 40:g,t:-	†	c	d	e	f	+	g	*	<i>Salmonella</i> spp.
8.	<i>S. enterica</i> subsp. <i>salamae</i> 38:b:1,2	†	c	d	e	f	+	g	*	<i>Salmonella</i> spp.
9.	S.I.6,7:k:-	†	c	d	e	a	+	g	*	<i>E. coli</i>
10.	S.I.6,7:k:-	+++	c	d	e	a	+	g	*	<i>Salmonella</i> spp.
11.	S. Cerro	++	a	a	a	a	+	h	*	<i>E. coli</i> inactive
12.	<i>S. enterica</i> subsp. <i>diarizonae</i> 61:k:1,5	+++	a	a	a	a	+	h	*	

<sup>a</sup> Typical *Salmonella* colonies for the medium tested. <sup>b</sup> Pink transparent colonies with large skirt and a very small black center like a ‘pinhead’. <sup>c</sup> Transparent colonies surrounded by yellow halos without black centers. <sup>d</sup> Red colonies (lactose-positive), without black centers. When co-cultured with typical *Salmonella* isolates, they progressively (in 30 h) exhibited the capability of producing H<sub>2</sub>S, as well as appearing as yellow colonies (lactose-negative), thus giving the appearance of typical *Salmonella* spp. <sup>e</sup> Yellow to greenish-yellow colonies and media, converted to red-colored colonies and red medium when co-cultured with typical *Salmonella* isolates. <sup>f</sup> Transparent colonies. <sup>g</sup> Yellow butt/slant and H<sub>2</sub>S for the first 24 h, but in 48 h the butt changed to red. <sup>h</sup> Yellow butt/slant and very little H<sub>2</sub>S production. † very little growth. ++ intermediate growth. +++ very good growth. ++++ excellent growth, the media turned white. \* Positive reaction for O and H antigens with polyvalent antisera. S.I: *S. enterica* subsp. *enterica*.



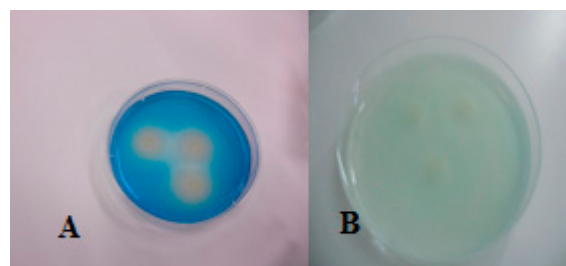


**Figure 3.** Typical *Salmonella* isolate (red arrows) on SS (a) and TSI (b) media co-cultured with (i) *Salmonella* H<sub>2</sub>S (–) (strains 17, 50/yellow arrows) and (ii) *E. coli* (strains 30, 113/green arrows) after 36 h incubation.

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 strains had a typical reaction when tested on TSI medium, while the last gave a yellow butt/slant and very little H<sub>2</sub>S production, also showing weak growth on MSR/V compared to the other three strains.

*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 a yellow butt/slant on TSI agar with very little H<sub>2</sub>S production. Interestingly, its biochemical profile given by MicrogenTMGnA+B-ID was ‘inactive *E. coli*’. *S. enterica* subsp. *diarizonae* 61:k:1,5 gave a red butt/yellow slant and very little H<sub>2</sub>S production on TSI. Both isolates showed weak growth on MSR/V but strong agglutination with the polyvalent antisera.

Finally, of the 615 samples cultured on MSR/V 59 that 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 4).



**Figure 4.** (A) *E. coli* on MSR/V; the medium does not inhibit the growth of the microorganism. (B) *Salmonella* spp. on MSR/V; the medium has a whitish color.

### 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) that were eventually identified as *Escherichia coli* (12 isolates), *Citrobacter freundii* (3 isolates), *Trabulsiella guamensis* (2 isolates), and *Klebsiella ozanae* (1 isolate). These showed little to very good growth on MSR/V agar (Table 2).

**Table 2.** Enterobacteriaceae resembling *Salmonella*: color of colony and media utilization.

A/A	ISOLATE	MSRV	XLD	SS	BG	SCA	H <sub>2</sub> S	TSI	<i>Salmonella</i> ANTISERA
8.	<i>E. coli</i>	(–)	a	a	a	f	(+)	p	O(+), H(–)
21.	<i>E. coli</i>	††	m	r	a	f	(–)	s	light (+) for O and H antigens
30.	<i>E. coli</i>	†	x	d	o	f	(–)	s	O(+), H(+)
48.	<i>E. coli</i>	††	a	a	a	f	(+)	p	O(+), H(–)
52.	<i>E. coli</i>	†††	a	a	a	f	(+)	a	O(+), H(+)
76.	<i>E. coli</i>	†††	b	r	a	f	(+)	p	O(+), H(–)
77.	<i>E. coli</i>	†††	a	a	a	f	(+)	p	O(+), H(–)
80.	<i>E. coli</i>	††††	a	a	a	f	(+)	p	light (+) for both O and H antigens
81.	<i>E. coli</i> inactive	††	a	a	a	f	(+)	p	light (+) for O and H antigens
82.		††	b	a	a	f	(+)	p	light (+) for O and H antigens
88.	<i>E. coli</i> (indole negative)	††	y	t	o	f	(+)	p	light (+) for O and H antigens
94.	<i>E. coli</i>	†	w	t	o	f	(+)	p	O(+), H(–)
75.	<i>Citrobacter</i> <i>freundii</i> (indole negative)	†††	y	d	o	f	(+)	p	light (+) for O and H antigens
83.	<i>Citrobacter</i> <i>freundii</i>	†††	b	d	e	f	(+)	p	light (+) for O and H antigens
93.	<i>Citrobacter</i> <i>freundii</i>	†††	b	a	a	f	(+)	p	light (+) for O and H antigens
4.	<i>Trabulsiella</i> <i>guamensis</i>	††	k	a	o	f	(+)	p	light (+) for O and H antigens
90.	<i>Trabulsiella</i> <i>guamensis</i>	††	y	t	o	f	(+)	p	O (light +), H(–)
73.	<i>Klebsiella ozanae</i>	††	z	a	o	f	(+)	p	O, H(–)

<sup>a</sup> Typical *Salmonella* colonies for the medium tested. <sup>f</sup> Transparent colonies. <sup>m</sup> Transparent pink colonies without black centers/red media. <sup>p</sup> Yellow butt/red slant, H<sub>2</sub>S (+). <sup>r</sup> Lactose-negative colonies without black centers. <sup>s</sup> Yellow butt/red slant, H<sub>2</sub>S (–). <sup>b</sup> Pink transparent colonies with large skirt and a very small black center like a ‘pinhead’. <sup>d</sup> Red colonies (lactose-positive), without black centers. When co-cultured with typical *Salmonella* isolates, they progressively (in 30 h) exhibited the capability to produce H<sub>2</sub>S, as well as appearing as yellow colonies (lactose-negative) and giving the appearance of typical *Salmonella* spp. <sup>e</sup> Yellow to greenish yellow colonies and media, converted to red coloured colonies- red medium when co-cultured with typical *Salmonella* isolates. <sup>w</sup> Large black colonies surrounded by reddish halos with yellow medium. <sup>x</sup> Yellow colonies (24 h) converted to red colonies (30 h). <sup>y</sup> Yellow medium/colonies with black centers. <sup>z</sup> Yellow medium/colonies. <sup>†</sup> Lactose-positive colonies with black centers. <sup>o</sup> Yellow to greenish-yellow colonies and media. <sup>†</sup> very little growth. <sup>††</sup> intermediate growth. <sup>†††</sup> very good growth. <sup>††††</sup> excellent growth, the media turned white. (+) positive reaction, (–) negative reaction

Specifically, *E. coli* isolates produced pink transparent colonies with or without ‘pin-head’ 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<sub>2</sub>S production and changed to yellow colonies (lactose-negative) after 48 h 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<sub>2</sub>S. The remaining two produced yellow butt/yellow slant/H<sub>2</sub>S (+)

(Figure 3). Interestingly, seven of them agglutinated partially 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 produced lactose-negative colonies with a black center. All of them showed colorless colonies on SCA and yellowish colonies on BG agar. They had yellow butt/red slant/H<sub>2</sub>S (+) 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 of the media that were evaluated and gave light agglutination with the polyvalent *Salmonella* antisera but 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, and transparent colonies on BG and gave a yellow butt/slant and a 'trace' of H<sub>2</sub>S production on TSI.

#### 4. Discussion

A wide range of methodologies are available for the bacteriological isolation and characterization of *Salmonella*, which demands 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 with existing bacteriological methods and the need for faster reliable results promote research into the development of molecular methods. However, for fecal and environmental samples derived from pigs, molecular methods are still in the early stages of development with regard to giving consistent results. For now, the adoption of new methods in routine laboratory monitoring control programs is a compromise between improved sensitivity, specificity and selectivity, 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 programs.

Thus, the growing availability of studies held periodically to determine the sensitivity of the various new culture media show that some perform better than others. Their performance is, perhaps, influenced by the kind of sample (e.g., food, feed, feces, environmental sources) or the species of the pathogen [32] infecting swine, with no culture method likely 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 MSR/V used for enrichment of swine feces improving recovery because it inhibits growth of most fecal bacteria species, increasing in this way the sensitivity of XLD and BGA agars [12,20,36]. Their sensitivity after the use of MSR/V 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 MSR/V showed extensive swarming indicative of *Salmonella*'s migration through the MSR/V 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 MSR/V 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 suspected microbial burden, which may also help 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 (MSR/V) as their inability to grow on this medium was confirmed. MSR/V medium had, perhaps, a killing



effect on this isolates that affected the prevalence of *Salmonella* spp. in an animal population or food stuff. This 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.

It was also evident that the choice of differential plating culture media significantly affects recovery. This 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 the use of chromogenic media makes the recognition of most salmonellae easier, the present study showed that they fail in cases with rarer *Salmonella* serovars. These serovars could come to be of epidemiological importance in later times if they are not accounted during control programs. Thus, failure of culture methods to recognize some *Salmonella* serovars leads 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. Since 1887, *E. coli* has been 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 characterize 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 for these to be accounted for 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 the growth of bacteria, such as *Klebsiella* spp., which carry the lactose (lac) operon on a plasmid or a chromosome [49,50].

Another important taxonomic characteristic of Enterobacteriaceae is the production of H<sub>2</sub>S. *Citrobacter*, *Proteus*, and *Salmonella* are major H<sub>2</sub>S-producing genera. H<sub>2</sub>S 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<sub>2</sub>S. However, this characteristic may be absent in environmental strains of *Salmonella* spp. due to mutations [53,54]. One pathway of H<sub>2</sub>S production is through the reduction of thiosulfate mediated by enzymethiosulfate reductase [51,55,56]. However, there are some problems in the interpretation of H<sub>2</sub>S production needing further consideration. Specifically:

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

- (ii) The commercial diagnostic media may also contain fermentable carbohydrates, resulting in the masking of H<sub>2</sub>S 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 H<sub>2</sub>S-producing enzymes and its H<sub>2</sub>S-producing rate [51,55,57]. Some consider that the ability of an isolate to produce H<sub>2</sub>S is plasmid mediated [58–62], although the nature of the plasmid differs from isolate to isolate. This could be the reason that lactose-positive *Salmonella* spp. appeared giving negative (–) H<sub>2</sub>S production on XLD and SS agars, possibly influenced by fermentation [63]. In the present study, non-H<sub>2</sub>S-producing *Salmonella* and *E. coli* isolates were stimulated to produce H<sub>2</sub>S when cultured side by side with H<sub>2</sub>S-producing isolates. Perhaps, the ‘masking’ of H<sub>2</sub>S was overcome in synergy when lactose-negative and strong H<sub>2</sub>S-producing isolates were cultured side by side. However, false-negative results decrease the recovery of *Salmonella* spp. if ISO recommendations are strictly followed, which influences its diagnostic value.

Of similar diagnostic importance is the isolation of *E. coli* strains producing H<sub>2</sub>S and resembling *Salmonella* on XLD. The inability of *E. coli* to produce detectable amounts of H<sub>2</sub>S on recommended culture media is a differential characteristic of the genus *Escherichia*, although H<sub>2</sub>S-producing variants have been isolated on numerous occasions [59,60,62,64–66]. Researchers suggest that the ability of *E. coli* to produce H<sub>2</sub>S is transferable between microorganisms in a similar way to the transfer of hemolysins, colicins, and plasmids for drug resistance and enterotoxin [60]. Although the isolation of H<sub>2</sub>S-producing variants of *E. coli* is infrequent [59] and their clinical significance remains unclear, their presence in specimens influences the diagnostic process due to misclassification. As for the role of H<sub>2</sub>S-producing microorganisms in the gut, one could think that they may synergistically reactivate the ‘masked’ ability of H<sub>2</sub>S-producing *Salmonella* spp., with the abundance of *E. coli* in the gut increasing their virulence.

Additionally, such diagnostic problems could negatively affect national schemes for 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 and 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 efficiently provide accurate results that are comparable between laboratories. The development of standard culture methods, as described by ISO 6579-1:2017, and the participation in proficiency quality assurance programs helps with harmonization between laboratories. However, there is not a single method that fulfills all criteria or is 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 and therefore the differentiation of *Salmonella* spp. from other Enterobacteriaceae sharing common biochemical and antigenic characteristics, while retaining cost-effectiveness. Reliability, ease of use, and costs are characteristics conventional selective and differential media as compared to the newer chromogenic ones not yet established. The use of two conventional selective media with different selective and differentiating properties in conjunction with the experience of the bench technician is likely to help with the detection of atypical strains, like the lactose-fermenting strains, which in most cases are discarded as they are not considered 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 strains 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, G.E. and A.R.B.; methodology, G.E. and A.R.B.; investigation, G.E. and A.R.B.; writing—original draft preparation, G.E. and A.R.B.; software, N.S.; resources, N.S.; supervision, N.S. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research received no external funding.

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** Data is contained within the article.

**Conflicts of Interest:** The authors declare no conflicts of interest.

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