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Summary

Shellfish frequently host pathogenic bacteria involved in toxic food infections, in particular *Salmonella* whose surveillance has to be undertaken carefully. The conventional method used for *Salmonella* detection is neither fast nor simple. A new rapid method reducing the duration of pre-enrichment and using Rambach agar has been compared with the conventional method of detection. The comparison included 133 samples at duplicate temperatures of 37°C and 42°C. The new protocol appeared more efficient than the reference technique in recovering *Salmonella* from bivalves (respectively 28 and 17 positive results) and is faster and simpler. The sensitivity of the isolation step on Rambach agar was satisfactory (under 10^5 *Salmonella* per ml). In addition, the validity of this medium in direct enumeration of *Salmonella* from shellfish has been demonstrated.

Key words: Salmonella; Shellfish; Rapid method; Rambach agar

Introduction

Among foodborne outbreaks of bacterial origins *Salmonella* appears as the major causative agent in many countries [1–3]. Its occurrence in the environment and importance in public health is increasing dramatically [4]. Dissemination in the environment occurs widely via water, and coastal seawater is often contaminated by sewage of human and animal origin [5]. Shellfish harvested in coastal areas consequently are in contact with this pollution. Some particular aspects of bivalve biology and shellfish consumption emphasize this contamination [6]. As a consequence, the presence of *Salmonella* in shellfish, both at harvesting and marketing stages are often reported [7] and *Salmonella* survey of commercial shellfish should be

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liquid) were collected and then diluted 1/3 in saline peptone water (SPW) and then homogenized in a Waring Blender for 30 to 40 s at 15000 rpm.

Salmonella detection

The presence of *Salmonella* in 25 g of bivalve content was investigated in parallel by the conventional reference method and by the rapid method tested here (short pre-enrichment-Rambach agar).

Conventional method. 125 ml of homogenenate (corresponding to 25 g of bivalve content) were inoculated for pre-enrichment in 125 ml of double strenght buffered peptone water (BPW) and incubated at 37°C.

After 16 to 24 h the pre-enrichment culture was transferred into two Rappaport-Vassiliadis enrichment media (R10) (Merck 10236) with a ratio of 1/100 (0.1 ml in 10 ml). One of the tubes was incubated at 37°C, the other one at 42°C for 24 h.

A loopful of these two enrichments was then streaked onto brillant green phenol Red Lactose Sucrose agar (BPLS) (Merck 7232) and the plates were incubated for 24 h at 37°C.

Characteristic colonies (bright red) were selected and analysed by API 20E identification strips. Strains identified as *Salmonella* were further characterised by serological typing.

Rapid method. 25 g of the same bivalve sample were submitted in parallel to the rapid method (125 ml of homogenate plus 125 ml of double strenght pre-enrichment medium).

The pre-enrichment was performed in the Salmosyst medium (Merck N°10153). After incubation of this pre-enrichment medium (peptone carbonated water) for 6–8 h at 37°C, a 10 ml aliquot was sampled and a selective tablet of Salmosyst complement (potassium tetrathionate, bile, brilliant green, calcium carbonate) (Merck 10141) was added to this aliquot. Two different tubes were prepared for incubation at 37 and 42°C for 18 h.

A loopful of these enrichments was then streaked onto Rambach agar. After incubation for 18 h at 37°C, the presumptive presence of *Salmonella* was noted and pre-identified *Salmonella* colonies were confirmed by API 20E and further serological identification.

Sensitivity of Salmonella detection on Rambach agar

To assess the lower limit of the detection by isolation on Rambach agar, the following reconstruction experiment was conducted: 100 ml of enriched Rappaport-Vassiliadis (RV) medium were prepared according to the conventional method, from a mixture of 3 bivalve samples which had previously given negative response to *Salmonella* detection.

After incubation this enriched culture was distributed among 11 tubes labelled 0 (blank) and A–J.

In parallel, a suspension of *Salmonella agona* (recently isolated from mussels) was prepared. This strain was cultivated 24 h on a Nutrient agar slant, suspended in Saline Peptone Water, centrifuged and re-suspended. The bacterial density of the final

suspension was expected to be in the range of 10^9 ml^{-1} (optical density at 650 nm = 1), and its actual concentration was obtained by enumeration on Nutrient agar.

The tubes labelled A to J were supplemented with this *Salmonella* suspension to obtain *Salmonella* densities ranging from 6×10^2 ml^{-1} to 1.2×10^8 ml^{-1}(values given in Table 4).

Isolation onto Rambach agar was performed from these 11 tubes by 3 different technicians (I, II, III) in parallel.

The presence of characteristic red colonies after incubation at 37°C for 24 and 48 h was reported.

Validity of Rambach agar in direct enumeration of Salmonella

The validity of Rambach agar for direct enumeration of *Salmonella* was tested with 3 different materials.

Bacterial suspension

The *Salmonella agona* suspension prepared for testing the sensitivity of Rambach agar (see above) was used for direct enumeration by spread plate method onto nutrient agar on the one hand, and Rambach agar on the other hand (10 plates per dilution).

Rappaport Vassiliadis enrichment. Enumeration by direct plate method onto Rambach agar was undertaken from tube D in the procedure previously presented for sensitivity testing. This tube was therefore an enriched culture from *Salmonella*-free bivalves supplemented by *Salmonella agona* (with a *Salmonella* concentration of 1.2×10^6 ml^{-1}).

Mussel samples. A direct enumeration by spread plate method on Rambach agar was performed, on the one hand from bivalve suspensions supplemented with *Salmonella*, and on the other hand from shellfish artificially contaminated by *Salmonella*.

Shellfish homogenates were prepared, in this case, by diluting bivalve content in SPW 1/3, and mixing for 2 minutes in a Stomacher homogenizer in a filter bag to obtain filtered suspensions more adapted to surface inoculation [11]. One part of this homogenate was supplemented (in the ratio 1/100) with a *Salmonella agona* suspension, and another part with *Salmonella enteritidis*, to obtain *Salmonella* densities approaching 10^2 per ml of homogenate. The fecal coliform contamination of these shellfish samples was assessed in parallel by the usual MPN procedure [12].

A batch of mussels was artificially contaminated in a seawater tank by a *Salmonella agona* suspension for 2 h. The *Salmonella* concentration in the seawater tank was 2.4×10^4 ml^{-1} during the contamination period.

Mussels were then removed from the tank and stored in the open air at 10°C for 8 days. Before storage a sample was taken and prepared for bacteriological analysis according to the procedure presented above.

A direct enumeration from this mussel homogenate was performed on the one hand onto Rambach agar, and on the other hand onto Marine agar (Difco N°2216).

A second similar enumeration was performed after 8 days air storage.

Rambach agar plates were incubated at 37°C for 24 h and marine agar plates were incubated at 30°C for 72 h.

Results

Comparison between conventional and rapid method

The first aim of this study was to compare the rapid method (RM) with shortened pre-enrichment and Rambach agar isolation, and the conventional method (CM) with buffered peptone water, Rappaport-Vassiliadis and BPLS. The second aim was to compare for these two methods, the efficacy of two temperatures (37° and 42°C) during the enrichment step.

The general responses obtained from these 133 paired detections are presented in Table 1.

During this survey of 133 bivalve samples, 31 samples (23.3%) presented positive responses for *Salmonella* presence in 25 g of bivalve content.

Among these positive results, only 14 were obtained by both methods, while 3 were obtained by the conventional method only, and 14 were obtained by the rapid method only.

The new rapid method appears therefore as more efficient in *Salmonella* recovery in the present survey. The rapid technique yielded 28 positive samples (21.3%), whereas the conventional method yielded only 17 positive results (12.8%).

The significance of the difference between the two methods may be assessed by a

TABLE 1

Distribution of positive and negative *Salmonella* detections as a function of the method

	Rapid method (RM)		
	positive	negative	total
Conventional method (CM)			
Positive	14	3	17
Negative	14	102	116
Total	28	105	133

TABLE 2

Presence and confirmation of *Salmonella*-like colonies after the isolation step

	Number of samples	Samples with suspect colonies	Samples with negative suspects	Samples with confirmed *Salmonella*
Conventional method (BPLS agar)	133	31	14	17
Rapid method (Rambach agar)	133	28	0	28

non-parametric statistic test: the Mac Nemar test [13], based upon numbers of samples giving opposite results with the two methods.

The value of the test in the present case is 2.67 ($p = 0.003$.) showing that the difference between the two methods is highly significant.

The purpose of the new method was to obtain a rapid response for the presence of *Salmonella* in bivalve samples. The results presented in Table 2 indicate that a good preliminary response can be obtained after the isolation step.

The totality of Rambach agar plates showing characteristic colonies have been confirmed as positive after biochemical confirmation. Thus, in this method the preliminary results, obtained within 48 h only, are similar to those obtained after complete detection.

TABLE 3

Detail of serotypes isolated, in function to the bivalve group, the technique and the temperature during the enrichment step

Sample species	Conventional method		Rapid method	
	37°C	42°C	37°C	42°C
Mussels			*S. typhimurium*	
Mussels			*S. panama*	*S. panama*
Cockels			*S. panama*	*S. panama*
Oysters			*S. bredeney*	
Mussels	*S. bredeney*	*S. bredeney*	*S. bredeney*	*S. bredeney*
Oysters			*S. bredeney*	*S. bredeney*
Oysters			*S. taksony*	*S. taksony*
Cockles		*S. bredeney*		*S. bredeney*
Cockles		*S. bredeney*	*S. bredeney*	*S. bredeney*
Mussels		*S. bredeney*	*S. bredeney*	*S. bredeney*
Mussels			*S. panama*	*S. panama*
Mussels	*S. agona*	*S. agona*	*S. agona*	*S. agona*
Mussels	*S. agona*	*S. agona*		
Oysters			*S. indiana*	*S. indiana*
Mussels	*S. saint paul*	*S. saint paul*	*S. saint paul*	*S. saint paul*
Oysters		*S. lille*	*S. lille*	*S. lille*
Mussels		*S. panama*	*S. panama*	*S. panama*
Mussels		*S. schwarzengrund*		
Cockles			*S. bredeney*	*S. bredeney*
Cockles		*S. bredeney*	*S. bredeney*	*S. bredeney*
Mussels			*S. meleagridis*	*S. meleagridis*
Mussels			*S. brandenburg*	*S. brandenburg*
Oysters			*S. panama*	*S. panama*
Cockles			*S. bredeney*	*S. bredeney*
Mussels	*S. typhi*	*S. typhi*	*S. typhi*	*S. typhi*
Cockles		*S. panama*		
Mussels		*S. lille*	*S. lille*	*S. lille*
Mussels	*S. panama*	*S. panama*	*S. panama*	*S. panama*
Cockles		*S. montevideo*	*S. montevideo*	*S. montevideo*
Cockles				*S. tarshyne*
Mussels		*S. panama*	*S. panama*	*S. panama*
Number of positive results	6	17	26	26

The *Salmonella* positive responses are detailed in Table 3. Among the 31 positive detections, 16 were obtained in mussel samples, 9 in cockles and 6 in oysters.

The most frequently isolated serotypes were respectively *S. bredeney* (9), and *S. panama* (7). *S. typhi* was isolated from one mussel sample.

This presentation allows a more detailed analysis of the results in function to the technique and the enrichment step temperature. In the conventional method with RV enrichment medium, the growth temperature of 42°C is confirmed as much better than a temperature of 37°C, and is generally recommended.

On the contrary, in the rapid method, with tetràthionate medium the same rates of positive results are obtained after 37 or 42°C. However in some cases, a positive result is obtained only with one of the two temperatures.

Sensitivity of the isolation on Rambach agar

Isolation on Rambach agar was performed in parallel by three different operators from a series of enrichment cultures (prepared from a *Salmonella* negative mussel sample) which were supplemented with *Salmonella agona*. The *Salmonella* concentration in each tube was inferred from the concentration in the *Salmonella* suspension and the dilution titre. They are indicated in Table 4.

The presence or absence of charasteristic colonies after isolation from these tubes is indicated in Table 4. For each plate a visual estimation of frequency of these colonies (in percentage) has been done. In some cases typical bright red colonies appeared but were not isolated (NI).

The response of the isolation on Rambach agar did not differ markedly from one operator to another. Low *Salmonella* concentrations (tubes G and H) gave non-isolated colonies, but after a 48 h incubation the size of these colonies was sufficient to allow a new isolation onto Rambach agar which gave a high proportion of *Salmonella* colonies.

TABLE 4

Sensitivity of isolation step on Rambach agar

	Enrichment tube										
	A	B	C	D	E	F	G	H	I	J	O
Salmonella concentration (ml^{-1})	$1.2\ 10^8$	$1.2\ 10^7$	$6\ 10^6$	$1.2\ 10^6$	$6\ 10^5$	$1.2\ 10^5$	$6\ 10^4$	$1.2\ 10^4$	$1.2\ 10^3$	$6\ 10^2$	0
Presence of *Salmonella*-like colonies on Rambach agar											
I	+	+	+	+	+	+	NI	+	–	–	–
II	+	+	+	+	+	+	+	+	–	–	–
III	+	+	+	+	+	+	+	NI	–	–	–
Estimated percentage of *Salmonella*-like colonies	93	96	91	78	53	31	13	2	0	0	0
Expected percentage of *Salmonella*-like colonies*	99.5	95.2	90.9	66.6	50	16.6	9.1	1.9	0.2	0.05	–

*On the basis of equivalence of *Salmonella*-like and other colonies in tube E.

TABLE 5

Direct enumeration of *Salmonella* on Rambach agar in miscellaneous shellfish samples

	Shellfish homogenates									Artificially contaminated mussels	
	Mussels			Oysters			Cockles			Before storage	After 8 days storage
	NS	SA	SE	NS	SA	SE	NS	SA	SE		
Salmonella-like colonies (per 0.1 ml of homogenate)	0	12	10	0	16	6	2	15	6	56 ($2.8\ 10^3$ g^{-1})	94 ($4.7\ 10^3$ g^{-1})
Non-*Salmonella* colonies (per 0.1 ml of omogenate)											
B	1	1	0	0	1	0	151	193	184	0	0
PP	0	0	0	0	0	0	11	13	7	0	0
Fecal coliform MPN (per 100 g of bivalve content)	$4.5\ 10^2$			<90			$>7.2\ 10^4$			<90	–
Heterotrophic flora (marine agar 30°C) (per 1 g of bivalve content)	–			–			–			$7.95\ 10^6$	$7.50\ 10^7$

NS = non supplemented; SA = supplemented with *Salmonella agona*; SE = supplemented with *Salmonella enteritidis*; B = blue colonies; PP = pale pink colonies.

Under a concentration of 1.2×10^4 ml^{-1}(tube H) *Salmonella* were no more detected. Consequently, a positive response on Rambach agar can be obtained after pre-enrichment and enrichment stages leading to *Salmonella* concentrations reaching 10^4 to 10^5 ml^{-1}.

In this particular case an equal proportion of *Salmonella* colonies and non-*Salmonella* colonies was obtained for tube E (6×10^5 *Salmonella* ml^{-1}). From this hypothesis of equivalence in tube E, the expected proportion in the other tubes could be inferred (Table 4). In most cases, the proportion of *Salmonella*-like colonies actually observed are very close to these expected values.

Direct enumeration of Salmonella on Rambach agar

Test with a bacterial suspension. A direct enumeration from a *Salmonella agona* suspension by spread plate method was undertaken in parallel on nutrient agar and Rambach agar. Very similar results were obtained by the two techniques:
Nutrient agar: 1.23×10^9 ml^{-1}; Rambach agar: 1.30×10^9 ml^{-1}.

A Student's *t*-test applied to the colony counts in the 10^{-6} dilution confirmed this equivalent efficiency of plating of the two media ($t = 0.8$; $0.3 < p < 0.5$: NS).

Test from an enrichment culture. A direct enumeration from a RV enrichment medium incubated 24 h at 42°C was tried. This enrichment was the tube D from the procedure for sensitivity of Rambach agar (see above). In this tube the *Salmonella* concentration was expected to be 1.2×10^6 ml^{-1} and representing 66% of colonies able to grow on Rambach agar.

After incubation for 24 h at 37°C, Rambach enumeration plates presented a majority of *Salmonella*-like colonies (in the same proportion as after isolation technique) associated essentially with coliform colonies (blue).

The *Salmonella* concentration obtained from this enumeration was 1.47×10^6 ml^{-1}. and was therefore in agreement with the concentration inferred from the concentration in the original suspension and the volume of inoculum in RV medium (1.2×10^6ml^{-1}) Thus the presence of this competitive flora in the enrichment medium does not interfere with *Salmonella* for a direct enumeration.

Test from bivalve samples. Salmonella direct enumeration was tried in supplemented shellfish homogenates, and in artificially contaminated mussels before and after a long air-storage period. The results are presented in Table 5.

In most situations, enumeration of *Salmonella*-like colonies was very easy, because non-characteristic colonies were very few and coliform-like (blue colonies). For oyster and mussel homogenates the incidence of associated flora was very low, and only a few non-characteristic colonies grew on Rambach agar. In cockle homogenate, however, non-*Salmonella* colonies were abundant, with a predominance of coliform colonies. This high contamination by coliform bacteria was confirmed by fecal coliform MPN. Nevertheless, in spite of this associated flora, *Salmonella* enumeration remained possible, and gave results similar to mussel and oyster homogenates.

Salmonella counts obtained with supplemented homogenates were therefore homogenous, and in agreement with the *Salmonella* densities in supplemented suspensions (respectively 1.49×10^3 ml^{-1} for *S. agona*,and 9.2×10^2 ml^{-1} for *S. enteritidis*). An enumeration from 0.6 ml of oyster homogenate (with *S. agona*) confirmed these counts. Even with this large inoculum, the bivalve tissue did not give interference, and *Salmonella* enumeration remained easy: 76 *Salmonella* colonies; 8 non-*Salmonella*-colonies.

Only in the case of highly contaminated cockles, were bright red colonies present in the non-supplemented sample. These two colonies (sampled after 24 h incubation) were identified respectively as *Citrobacter freundi* and fluorescent *Pseudomonas* group, and gave after 48 h an aspect different from *Salmonella* characteristic colonies.

Enumeration from artificially contaminated mussels indicated that even before storage the natural flora in this mussel sample reached a high level. However, this abundant flora did not interfere with *Salmonella* enumeration. On Rambach agar only *Salmonella*-like colonies were present and so easily counted.

After storage the mussels were badly spoiled (shells opened, ammonia odour, etc.), but the bacterial counts were not very different. The level of total heterotrophic flora had increased by one order of magnitude, and *Salmonella* concentrations increased slightly. As before storage, only *Salmonella*-like colonies were recovered on Rambach agar.

Discussion

The value of the new method tested here for detection of *Salmonella* in bivalves appears clearly. This method (which has been recently certified by AFNOR, the French institute for standardization) led to a rate of positive results much greater than the conventional method (respectively 21.3% instead of 12.8%).

These positive responses are not preliminary results mixed with some positive results such as the results obtained by other new methods. They are actual positive responses, as shown by biochemical confirmation and serological identification of the strains isolated. Consequently, a false positive rate cannot be calculated. The false negative rate observed is very low (3/133).

The difference between the two techniques is highly significant in the comparative study conducted here (133 paired samples). A more general study involving several laboratories of shellfish sanitation control is underway to confirm this first assessment.

The difference in the rates of *Salmonella* positive responses obtained by the new method, raises questions on the value of the reference method and also the values of the statistical data dealing with *Salmonella* prevalence as a function of time or geographic area. It would appear that these data may differ considerably according to the choice of the *Salmonella* detection method. The more reliable technique is the one leading to the higher confirmed positive rate. The reference method for *Salmonella* detection in bivalves is adapted from the more general method for detection in foods (ISO-6579). This method is not necessarily the more reliable for the specific conditions encountered in the detection from bivalves and more investigations will have to be conducted in this particular field.

Only the samples giving confirmed positive results in the Salmosyst-Rambach method had presented characteristic *Salmonella* colonies. Consequently, the preliminary results obtained after the isolation step and before biochemical characterization can be considered as a good indication obtained with good reliability and a low false positive rate within 48 h.

This short detection time is also the time required for most other new rapid methods quoted by Beumer et al. [8] in their comparative study. In contrast with these methods, further confirmation can be performed immediately on colonies.

The comparative study conducted here does not distinguish the relative efficacies of the enrichment steps and isolation step in the good *Salmonella* recovery observed with the rapid technique.

The pre-enrichment and enrichment steps are essential in all *Salmonella* detection techniques. The need for the pre-enrichment stage which is essential to recover sublethally damaged bacteria, is generally recognized [14,15]. The value of the miscellaneous enrichment media available has been widely discussed. Most of the recent studies confirm the good performance of the Rappaport-Vassiliadis (RV) enrichment medium, which had been reported by these authors [16]. These good results have been observed for various samples, as human feces, farm animals' and seabird feces, and food products [17–19], and are reviewed by D'Aoust [1].

More precisely, Beumer et al. [8] have compared the kinetics of *Salmonella* and competitive Enterobacteriaceae during the enrichment stage. The superiority of the

RV medium, based on both a shorter generation time of *Salmonella* and a rapid decrease of competitive flora, appears clearly. Another comparative study [20] underlines the role of the competitive flora in the enrichment step, and indicates a higher toxicity of RV medium against stressed *Salmonella* cells. Furthermore the RV medium has been considered as problematic in the presence of an abundant competitive flora [21].

Until now, studies testing the relative efficiency of these enrichment media, in the particular case of marine bivalves, which possess a rather specific bacterial flora [6], have not been undertaken.

The transfer volume into the enrichment medium differs greatly between these two methods and may influence their relative performances, although an equivalent sensitivity of 1 ml and 0.1 ml transfer has been demonstrated [22].

In parallel, the performances of agar plating media have been investigated [1,23]. The qualities stressed by Rambach [9] for his new isolation medium were mainly:
- easier differentiation of *Salmonella* colonies by propylene glycol acidification;
- high sensitivity leading to the absence of false positive colonies;
- good agreement with the reference medium (BPLS).

The first two qualities appear clearly in the present comparative study. In contrast, some inconsistency appears between the conventional and the rapid new method, related either to the isolation medium or the enrichment medium. This specific question remains to be examined.

A high selectivity of Rambach agar has been observed in the comparative study presented here. Our *Salmonella* supplementation test shows that the ratio between *Salmonella* and competitive flora followed the dilution indicating that the quasi-totality of the *Salmonella* present in the enrichment media are recovered after isolation. The sensitivity limit is therefore the volume of the loopful of enrichment medium sampled for isolation.

The low incidence of the competitive flora explains the low threshold obtained for *Salmonella* detection (10^4–10^5 ml^{-1}), lower than the level generally reached after the enrichment stage [8].

This level is, in part, related to the *Salmonella* concentration in the original sample. The high sensitivity of Rambach agar demonstrated here could therefore represent one of the reasons of the good recovery found in the comparative study.

The high selectivity of Rambach agar also turned out to be quite favourable in direct enumeration. As expected, no difference appeared between this medium and nutrient agar in the enumeration of a *Salmonella* suspension. A test with a supplemented enrichment demonstrated that enumeration in the presence of a high associated flora is feasible without a reduction in *Salmonella* counts.

The high selectivity of the medium appeared particularly in direct enumeration from bivalves. Even in highly contaminated bivalves with the presence of a high fecal coliform contamination and with an associated flora approaching 10^8 ml^{-1}, *Salmonella* enumeration was performed easily without interference by the contaminating flora.

The use of large Petri dishes (140 mm) allows the enumeration from 0.2 gram of bivalve content and therefore the lower limit for direct *Salmonella* enumeration from bivalves may be estimated at few *Salmonella* units in 1 g of bivalve.

However, these densities are rarely encountered under normal conditions and a semi-quantitative detection with enrichment stages remains necessary in the regular *Salmonella* survey.

Nevertheless, the direct enumeration technique may be very useful for instance in experimental studies which have to be conducted to investigate the fate of *Salmonella* in bivalves (or other products) under various conditions. Until now, most of these studies have been undertaken only with indicator bacteria for methodological reasons. Other selective agar media have been proposed for direct enumeration of *Salmonella* [24,25] and one of them was used in a study dealing with *Salmonella* behaviour in shellfish [26]. The direct enumeration on Rambach agar appears from the present study as an interesting alternative in this prospect.

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