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Comparison of rapid cultural methods for the detection of *Salmonella* species.

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28 Abstract

29 Three newly developed rapid cultural methods (Rapid *Salmonella*, Precis™ *Salmonella*,
30 IBISA *Salmonella*) for the detection of *Salmonella* spp. were compared to a reference
31 method. All methods performed comparably on inclusivity/exclusivity testing. Similar limits of
32 detection were observed for all methods with milk, cocoa and bouillon matrices. Some tea
33 varieties appeared to disturb the normal color formation of all selective agars tested.

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35 Keywords: rapid cultural methods, *Salmonella* detection, food matrix, inclusivity& exclusivity

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42 1. Introduction

43 Traditional culture methods for the isolation and identification of *Salmonella* from food involve
44 enrichment in a primary non-selective broth, such as lactose broth or buffered peptone water
45 (BPW). This is followed by secondary selective enrichments in two media chosen from
46 Rappaport-Vassiliadis (Soya) broth (RV(S)), selenite cystine broth and/or a tetrathionate
47 broth. After plating on two selective agars, the suspect colonies are confirmed biochemically
48 and serologically (ISO 6579:2002; BAM, 2011). The complete procedure requires at least
49 three days to obtain a negative result and up to seven days for a confirmed positive result.
50 Cultural methods have the advantage that they are capable of detecting viable cells, however
51 the testing of large sample numbers is time consuming and labor intensive (Maciorowski et
52 al., 2006). Production lots often need to be held in storage for several days during
53 *Salmonella* testing. Therefore, the demand exists for faster yet still reliable and sensitive
54 methods. The close relationship with other genera such as *Escherichia coli*, *Cronobacter* and
55 *Citrobacter* spp. presents another challenge to the cultural detection of *Salmonella* spp..
56 These organisms compete in the enrichment media and/or can cause false positive reactions
57 in *Salmonella* tests. Particularly in foods with a high initial background flora, low levels of
58 *Salmonella* might be overgrown by the competing bacteria. (D'Aoust et al., 1992; Bennett et
59 al., 1999) Newly developed cultural methods combine a single enrichment in a selective
60 broth at an elevated temperature followed by isolation on a chromogenic agar. Background
61 organisms are suppressed by the selective agents in the broth and agar and/or can be
62 differentiated from *Salmonella* via the color reaction on the selective agar. Elimination of the
63 non-selective enrichment step means the risk of overgrowth of *Salmonella* by competitors is
64 reduced and that a negative result can be obtained in 44-48 hours.

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68 2. Material and methods

69 2.1. Methods

70 The inclusivity/exclusivity and ability to recover *Salmonella* from difficult food matrices was
71 evaluated for the following rapid cultural methods: Rapid *Salmonella* (Bio-Rad, Marnes-la-
72 Coquette, France), Precis™ *Salmonella* (Oxoid, Pratteln, Switzerland) and IBISA *Salmonella*
73 (AES chemunex, Bruz cedex, France). Rapid *Salmonella* and IBISA *Salmonella* include a
74 supplement to BPW, which contains both nutrients to resuscitate injured *Salmonella* and
75 inhibiting substances against competitor organisms. The Precis™ *Salmonella* method
76 includes the ONE Broth-*Salmonella*, which is a complete selective enrichment medium. After
77 the enrichment in the respective broths, aliquots are streaked on Rapid`*Salmonella* agar,
78 Brilliance™ *Salmonella* agar and IBISA agar. *Salmonella* can be differentiated from non-
79 target bacteria based on colony color and positive results are confirmed with a latex test. The
80 reference method used was based on ISO 6579: 2002 using BPW, RVS, XLD and ChromID
81 (bioMérieux, Marcy l'Etoile, France) for the detection of *Salmonella* species, this version of
82 the standard method has been validated using ISO 16140 (MicroVal certificate number
83 MV2007-LR06).

84 2.2 Strains

85 Forty-nine *Salmonella enterica* subsp. *enterica* strains and 32 non-*Salmonella* strains were
86 used to determine inclusivity (sensitivity) and exclusivity (specificity) in the different media.
87 The strains were chosen to provide a variety of serovars/species commonly found in food
88 products and included strains that had given unexpected results in previous method
89 evaluation studies. All strains were natural isolates obtained from our in-house collections.

90 2.3 Determination of doubling times

91 For the investigation of doubling times in the four enrichment broths (ONE broth-*Salmonella*,
92 BPW+ Rapid`*Salmonella* supplement, BPW+ IBISA *Salmonella* supplement, BPW and RVS),
93 overnight cultures of six *Salmonella* strains, as well as one strain each of *Cronobacter*

94 *sakazakii*, *Shigella flexneri*, *Escherichia coli* and *Citrobacter freundii* were grown in Tryptic
95 Soy Broth (TSB, Oxoid) at 37 °C and serially diluted to 1: 10⁻⁶ in 9 ml of the recommended
96 broth for each method. A 400 µL aliquot of each dilution from 1: 10⁻² to 1: 10⁻⁶ was pipetted
97 into duplicate wells of a Bioscreen C honeycomb plate. The plates were incubated in a
98 Bioscreen C optical density reader (iLF bioserve e. K., Langenau, Germany) for 24 hours at
99 41.5 °C and the OD_{600nm} of the wells was measured every 15 minutes. The strains were
100 similarly prepared in BPW and were incubated in the Bioscreen C for 24 hours at 37 °C. The
101 doubling time for each strain was calculated using the regression equation for the linear
102 trendline.

103 2.4. Limit of detection

104 For the determination of the limit of detection (LOD), four different food matrices (skimmed
105 milk powder, sugar free cocoa, black tea and bouillon powder with Mediterranean herbs)
106 were artificially inoculated. The LOD_p is the contamination level (cfu/g) leading to a positive
107 result with a specified probability, p. The LOD_{50} and LOD_{95} were calculated for each method
108 with four different food matrices, specifying the smallest quantity of *Salmonella* that can be
109 detected with a probability of 50% or 95%, respectively. An overnight culture of *Salmonella*
110 Tennessee strain S511 was diluted to achieve inoculation levels of approximately 3 CFU/25
111 g, 1 CFU/25 g and 0.3 CFU/25 g, with six replicates per inoculation level. Two 25 g portions
112 of uninoculated sample served as negative controls and one positive control was inoculated
113 with approximately 10 CFU/25 g.

114 For the skimmed milk and sugar free cocoa powder matrices, 25 g of each food product was
115 enriched in 225 mL of the specific broth for each method. For the black tea and
116 Mediterranean bouillon powder matrices, 25 g of the sample was added to 900 mL of broth.
117 This higher dilution is routinely used in commercial testing laboratories as it has been found
118 to be necessary to prevent growth inhibition of the target organism from the herb, spices and
119 salt content of these matrices. For the rapid cultural methods, the enrichments were
120 incubated for 16-20 hrs at 41.5 °C before streaking 10 µL onto selective medium. For the

121 reference method, primary enrichment was carried out in BPW, with 100 mL of a 10 %
122 solution of non-fat dry milk (NFDP) added to the BPW for enrichment of cocoa powder. All
123 BPW enrichments were incubated for 16-20 hrs at 37 °C, before transfer of 0.1 mL to 10 mL
124 of RVS broth, followed by incubation at 41.5 °C for 18-24 hrs. A 10 µL aliquot was then
125 streaked onto selective agar.

126

127 Based on the qualitative results statistical analysis was carried out by application of the
128 EXCEL sheet PODLOD.xls (Wilrich & Wilrich, 2009). The LOD_{50} with confidence limits for
129 each method with each matrix were calculated (table 3).

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142 3. Results & Discussion

143 3.1. Inclusivity/ exclusivity

144 All methods performed with 100% specificity and sensitivity. Where growth of non-
145 *Salmonella* strains occurred, these could be differentiated from target *Salmonella* strains by
146 the colony colour (see table 1a &b). On the IBISA agar, some *Salmonella* serovars only
147 showed weak growth. Non-target organisms which showed growth similar to *Salmonella* in
148 the selective broths (*Cronobacter malonaticus* and *Cronobacter muytjensii*) did not grow on
149 the chromogenic agar plates of the three alternative methods.

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151 3.2. Growth rates in the selective broths

152 The growth rate of *Salmonella* in BPW, RVS and in broths from the Precis *Salmonella* and
153 IBISA *Salmonella* methods were found to be in a similar range (table 2). For all rapid method
154 broths the growth of *E. coli* and *S. flexneri* was inhibited. The doubling time of *C. sakazakii*
155 ranged from 18.62 minutes in BPW to 44.94 minutes in Rapid *Salmonella* selective broth.
156 RVS and Rapid *Salmonella* inhibited the growth of the *C. freundii* strain. Doubling times for
157 this strain in the other broths ranged from 25.04 to 41.15 minutes. *Cronobacter* and
158 *Citrobacter* are the main background organisms that pose problems for the isolation of
159 *Salmonella*. These organisms can generally grow on *Salmonella* selective agar, but do not
160 produce typical *Salmonella* colony morphologies. Therefore false positive colonies should not
161 be a cause for concern. However, the potential exists for false negative results due to
162 overgrowth of the target organisms from the competing flora.

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164 3.3 Detection limit in the food matrix

165 For the milk powder, cocoa powder and Mediterranean bouillon powder, all methods could
166 detect levels of contamination ranging from < 0.008- 0.144 CFU/g of product. For black tea

167 the LOD_{50} ranged from 0.051 to 0.219 CFU/g. When black tea was enriched with the
168 different broths and isolated on the selective agars, atypical colouration of the colonies made
169 it difficult to interpret the result on the plates (table 3).

170 Further analysis was performed with different tea varieties by overnight enrichment of 25 g in
171 900 mL BPW inoculated with approximately 10 CFU of *Salmonella* Congo S617. The
172 enrichment was then streaked on 6 different selective agars for presence/absence testing.
173 The selective agars were: XLD, BGA, chromID™ *Salmonella* (bioMérieux), Rapid *Salmonella*
174 (BioRad), Precis™ *Salmonella* (Oxoid) and IBISA *Salmonella* (AES chemunex). Two tea
175 varieties appear to contain ingredients, which disturb the normal color formation of all
176 selective agars tested.

177 In conclusion, newly developed rapid cultural methods present a time saving of 48 hours for
178 the cultural detection of *Salmonella* spp. in food samples. This saving is achieved by omitting
179 the non-selective enrichment step and using a fast latex agglutination test for confirmation of
180 presumptive positive colonies. The tested methods were as sensitive and specific as the
181 reference method when pure cultures of target and non-target strains were tested. Difficult
182 matrices such as black tea can pose a problem with false negative results due to atypical
183 colony colors.

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213 **Table 1:** Inclusivity results for each method evaluated.

<i>Salmonella</i> strains	N	Rapid	Precis	IBISA	Reference method	
		<i>Salmonella</i> (Magenta*)	<i>Salmonella</i> (Purple*)	<i>Salmonella</i> (Green*)	XLD (Black*)	ChromID (Pale pink/mauve*)
<i>Salmonella</i> Agona	1	+	+	+	+	+
<i>Salmonella</i> Albany	1	+	+	+	+	+
<i>Salmonella</i> Anatum	3	+	+	+	+	+
<i>Salmonella</i> Ank	1	+	+	+	+	+
<i>Salmonella</i> Bergen	1	+	+	+	+	+
<i>Salmonella</i> Bracknell	1	+	+	+	(clear)	+
<i>Salmonella</i> Cerro	1	+	+	+	+	+
<i>Salmonella</i> Congo	1	+	+	+	+	+
<i>Salmonella</i> Chittagong	1	+	+	+	+	+
<i>Salmonella</i> Denver	1	+	+	+	+	+
<i>Salmonella</i> Derby	1	+	+	+	+	+
<i>Salmonella</i> Enteritidis	2	+	+	+	+	+
<i>Salmonella</i> Ekotedo	1	+	+	+	(clear)	+
<i>Salmonella</i> Farmsen	1	+	+	+	+	+
<i>Salmonella</i> Gambe	1	+	+	+	(clear)	+
<i>Salmonella</i> Ibandan	1	+	+	+	+	+
<i>Salmonella</i> Infantis	2	+	+	+	+	+
<i>Salmonella</i> Kentucky	1	+	+	+	(clear)	+
<i>Salmonella</i> Kibi	1	+	+	+	+	+
<i>Salmonella</i> Kinondoni	1	+	+	+	+	+
<i>Salmonella</i> Limete	1	+	+	+	+	+
<i>Salmonella</i> Maritzburg	1	+	+	+	+	+
<i>Salmonella</i> Mbandanka	1	+	+	+ (weak)	+	+
<i>Salmonella</i> Napoli	2	+	+	+	+	+
<i>Salmonella</i> Nashua	1	+	+	+ (weak)	(clear)	+
<i>Salmonella</i> Oranienburg	1	+	+	+	(clear)	+
<i>Salmonella</i> Panama	1	+	+	+	+	+
<i>Salmonella</i> Plymouth	1	+	+	+	+	+
<i>Salmonella</i> Rissen	1	+	+	+	+	+
<i>Salmonella</i> Salamae	1	+	+	+ (weak)	(clear)	+
<i>Salmonella</i> Sundsvall	1	+	+	+	+	+
<i>Salmonella</i> Tennessee	2	+	+	+ (weak)	+	+
<i>Salmonella</i> Thompson	1	+	+	+	+	+
<i>Salmonella</i> Typhimurium	5	+	+	+ (weak)	(clear)	+
<i>Salmonella</i> Uno	1	+	+	+	+	+
<i>Salmonella</i> Virchow	1	+	+	+	+	+
<i>Salmonella</i> Wagadugu	1	+	+	+	+	+
<i>Salmonella</i> Wagama	1	+	+	+	+	+
<i>Salmonella</i> Wagenia	1	+	+	+	(clear)	+
Inclusivity %		100%	100%	100%		100%

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215 Footnotes: -, no growth, +, typical growth, + (weak), weak growth, N, number of strains tested

216 * expected typical colony color for *Salmonella* spp.

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Table 1b: Exclusivity results for each method evaluated. * expected typical colony color for *Salmonella* spp.

Organism	Rapid <i>Salmonella</i> (Magenta*)	Precis <i>Salmonella</i> (Purple*)	IBISA <i>Salmonella</i> (Green*)	Reference method	
				XLD (Black*)	ChromID (Pale pink/mauve*)
<i>Enterobacter hormaechei</i>	green	green	-	Atypical (yellow)	Atypical (turquoise)
<i>Shigella flexneri</i>	-	-	-	-	Atypical (white)
<i>Enterobacter asburiae</i>	-	green	-	-	Atypical (turquoise)
<i>Proteus mirabilis</i>	-	-	-	-	Atypical (beige)
<i>Providencia alcalifaciens</i>	-	beige	-	-	-
<i>Cronobacter turicensis</i>	dark blue	-	Purple	Atypical (yellow)	Atypical (dark blue)
<i>Hafnia alvei</i>	-	-	-	Atypical (clear)	Atypical (turquoise)
<i>Klebsiella oxytoca</i>	-	green	-	Atypical (yellow)	Atypical (blue green)
<i>Cronobacter sakazakii</i>	-	-	black	-	Atypical (green)
<i>Enterobacter cloacae</i>	-	-	-	-	-
<i>Cronobacter malonaticus</i>	-	-	-	Atypical (beige)	Atypical (blue)
<i>Cronobacter muytjensii</i>	-	-	-	Atypical (yellow)	Atypical (blue purple)
<i>Escherichia coli</i>	-	-	-	Atypical (yellow)	Atypical (turquoise)
<i>Escherichia coli</i>	-	-	-	Atypical (clear)	Atypical (turquoise)
<i>Serratia liquefaciens</i>	-	-	-	-	-
<i>Enterobacter amnigenus</i>	-	-	-	-	Atypical (turquoise)
<i>Cronobacter sakazakii</i>	-	-	-	Atypical (white)	Atypical (dark blue)
<i>Enterobacter asburiae</i>	-	green	-	Atypical (yellow)	Atypical (turquoise)
<i>Serratia plymuthica</i>	-	-	-	-	-
<i>Citrobacter freundii</i>	-	beige	-	Atypical (yellow)	Atypical (turquoise)
<i>Citrobacter freundii</i>	rosé	beige	-	Atypical (yellow)	Atypical (turquoise)
<i>Citrobacter freundii</i>	-	beige	-	Atypical (clear)	Atypical (turquoise)
<i>Citrobacter freundii</i>	-	-	-	-	Atypical (turquoise)
<i>Enterococcus faecalis</i>	-	-	-	-	-
<i>Micrococcus luteus</i>	-	-	-	-	-
<i>Pseudomonas aeruginosa</i>	-	-	-	-	-
<i>Pseudomonas fluorescens</i>	-	-	-	-	-
<i>Listeria innocua</i>	-	-	-	-	-
<i>Pantoea spp.</i>	-	-	-	-	-
Exclusivity %	100%	100%	100%	100%	

Footnotes: -, no growth,

* expected typical colony color for *Salmonella* spp.

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223 **Table 2:** Doubling time (minutes) in base broths for each isolation method.

Organism	Rapid	Precis	IBISA	BPW	RVS broth
	<i>Salmonella</i>	<i>Salmonella</i>	<i>Salmonella</i>		
<i>Salmonella</i>	35.3	27.3	28.4	21.9	29.7
(average +/- STDEV)	+/- 16.4	+/- 8.5	+/- 8.8	+/- 1.9	+/- 6.3
<i>Cronobacter sakazakii</i>	44.94	26.94	21.49	18.62	43.76
<i>Shigella flexneri</i>	-	-	-	24.43	-
<i>Escherichia coli</i>	-	-	-	15.66	-
<i>Citrobacter freundii</i>	-	36.20	41.15	25.04	-

Footnote: -, no growth

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252 **Table 3:** Limit of detection (CFU/g) with upper and lower 95 % confidence limits

Method	Milk powder		Bouillon powder		Black tea		Cocoa powder	
	<i>LOD</i> ₅₀	Confidence Limit	<i>LOD</i> ₅₀	Confidence Limit	<i>LOD</i> ₅₀	Confidence Limit	<i>LOD</i> ₅₀	Confidence Limit
Reference	0.039	0.018-0.084	0.009	0.004-0.020	0.110	0.054-0.224	0.057	0.027-0.120
Rapid <i>Salmonella</i>	0.144	0.070-0.296	0.039	0.017-0.089	0.062	0.030-0.129	0.082	0.040-0.168
Precis <i>Salmonella</i>	0.057	0.027-0.119	0.036	0.016-0.079	0.051	0.024-0.107	0.088	0.043-0.181
IBISA	0.024	0.011-0.056	0.007	0.003-0.015	0.219	0.100-0.477	0.068	0.033-0.142

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