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Evaluation of Rambach Agar for Detection of *Salmonella* Subspecies I to VI

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***Salmonella* strains belonging to subspecies I to VI were investigated for colony color when grown on Rambach agar. Most strains of *Salmonella* subspecies I, II, IV, and VI behaved as described. All strains of *Salmonella* subspecies IIIa, IIIb, and V produced β-D-galactosidase and blue-green colonies which could not be distinguished in color from *Escherichia coli* and other lactose-fermenting members of the family *Enterobacteriaceae*.**

Media that are selective for *Salmonella* species, such as desoxycholate-citrate agar, XLD agar, brilliant green agar, and galle-chrysoidin-glycerol agar (10) differ in the principles of selection they employ and also use biochemical properties of the organism for presumptive identification. Rambach (8) has described a medium which uses the ability of *Salmonella* species to produce acid from propylene glycol as an identifying characteristic. Additionally, β-D-galactosidase production by other members of the family *Enterobacteriaceae* is detected by reaction with the chromogenic substrate X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), which results in formation of blue-green colonies. The combination of colors resulting from the two reactions differentiates *Salmonella* species, with the exception of *Salmonella typhi*, from competing organisms. *Proteus* species and *S. typhi* appear as colorless colonies, *Citrobacter freundii* appears violet, and *Escherichia coli* and *Klebsiella* species appear as blue colonies.

The National Reference Center for Salmonellosis currently types *Salmonella* strains isolated from humans, animals, foods, and feeds by biochemistry, serology, antibiotic susceptibility pattern, phage typing, and plasmid profile. When using Rambach agar to check the purity of cultures received from district institutes, it was observed that some of the *Salmonella* subspecies did not form pink, red, crimson, or yellow to colorless colonies. Blue-green *Salmonella* colonies were seen; this unexpected color formation was probably due to β-D-galactosidase activity. Subsequently, a number of *Salmonella* strains of subspecies I to VI, characterized by their well-known different β-D-galactosidase activities, were checked on Rambach agar. The objective was to explore the possibility that Rambach agar is not reliable as a presumptive measure to recognize all of the *Salmonella* subspecies owing to the formation of blue-green colonies which may be overlooked as nonsalmonellae when they are members of certain subspecies.

Thirty-five strains of *Salmonella* subspecies I, II, IIIa, IIIb, IV, V, and VI obtained from various collections (World Health Organization Collaborating Center for Reference and Research on Salmonella, Paris, France; Nationale Referenzzentrum für Enteritiserreger, Hamburg, Germany; Statens Serum-institute, Copenhagen, Denmark; Robert Koch Institute and Robert von Ostertag Institute, Berlin and Wernigerode, Germany; Pasteur Institute, Paris, France) (Table 1) were examined. Additionally, 35 strains of *Salmonella* subspecies II to V recently isolated from different hosts (Table 2) were examined. All the strains were freshly cultivated before inoculation and incubation on Rambach agar. Antigenic types were determined by using specific sera (SIFIN, Berlin, Germany, and National Reference Center for Salmonellosis, Wernigerode, Germany).

β-D-Galactosidase testing was performed by using the *o*-nitrophenyl-β-D-galactopyranoside (ONPG) Reagnost test (Feinchemie Sebnitz GmbH, Sebnitz, Germany). Rambach agar (batch 10122) was kindly provided by Merck, Darmstadt, Germany. Rambach agar and the ONPG Reagnost test were controlled with *E. coli* NCTC 10418 and *S. typhimurium* LT1 M307, respectively. The color of *Salmonella* growth was observed from the whole inoculation streak and from single colonies after 24 h of incubation at 37°C.

The colors of *Salmonella* subspecies I colonies on Rambach agar were as described previously (1–3, 8). Most were pink, red, or crimson. Three of five *Salmonella* strains of subspecies II showed a blue streak and single colonies of blue to red (Table 1). All fresh isolated *Salmonella* subspecies II strains appeared as red or crimson colonies (Table 2). None of the 18 strains gave a positive ONPG reaction. All 10 strains of subspecies IIIa and all 15 strains of subspecies IIIb produced blue-violet streaks. Single colonies also appeared blue-violet. All 25 strains were positive for the ONGP test (Tables 1 and 2). Strains of subspecies IV grew as beige to colorless streaks, with single colonies appearing dark beige to red. All five strains of subspecies V showed colors similar to those of subspecies IIIa and IIIb. One of the two strains of subspecies VI grew as a blue streak and red-violet single colonies. The second strain behaved similarly to the strains of subspecies I.

Salmonella strains which are able to produce and release β-D-galactosidase, in particular subspecies IIIa, IIIb, and V, formed blue to blue-violet colonies and blue-green streaks. The percentages of the six subspecies giving an ONPG-positive reaction were as follows (5): subspecies I, 2%; subspecies II, 15%; subspecies IIIa, IIIb, and V, 100%; and subspecies VI, 44%. No strains of subspecies IV gave a positive ONPG reaction. The results explained the similarity in behavior of subspecies I and IV and also the occurrence of blue colonies within subspecies II, V, and VI.

The great majority of *Salmonella* strains isolated from a variety of sources belong to subspecies I. The serotypes of

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TABLE 1. Examination of *Salmonella* subspecies I to VI obtained from various collections on Rambach agar and by β-D-galactosidase (ONPG) reaction

Strain or subspecies	Antigen formula	ONPG test	Color on Rambach agar	
			Streak	Single colonies
S. gallinarum Ti 184	9,12:−:	−	Beige	None
S. abortusovis 18/65	4,12:C:1,6	−	Colorless to beige	None
S. abortusequi 835/81	4,12:−:e,n,x	−	Beige	Light red
S. choleraesuis 113/65	6,7:C:1,5	−	Beige	Light red
S. dublin 2230/91	9,12:g,p:−	−	Colorless	Red, light edge
S. typhimurium 870/92 LT2b/15	4,5,12:i:1,2	−	Pink	Pink, light edge
S. enteritidis 890/92 LT 4/6	9,12:g,m:−	−	Colorless	Orange, light edge
S. paratyphi A 1/96	2,12:a:−	−	Beige	Red, light edge
S. paratyphi B 7/93	4,5,12:b:1,2	−	Beige	Dark beige, light edge
S. typhi LT A	9,12,Vi:d:−	−	Beige	Red, light edge
Subsp. II no. 18	4,12:a:e,n,x	−	Blue	Blue and red, light edge
Subsp. II no. 69	4,12:d:e,n,x	−	Blue	Red-violet and Blue-violet
Subsp. II no. 81	4,12:e,n,x:1,5,7	−	Red, wrong growth	Red, wrong growth
Subsp. II no. 90	4,12:g,m,t:e,n,x	−	Pink	Pink, light edge
Subsp. II no. 1362	57:z_{42}:1,6:z_{53}	−	Blue	Blue
Subsp. IIIa no. 1000	18:z_4,z_{32}:−	(+)	Blue	Blue-violet
Subsp. IIIa no. 1329	53:g,z_{51}:−	+	Blue, wrong growth	Blue, wrong growth
Subsp. IIIa no. 1352	56:z_4,z_{23}:−	++	Blue	Blue
Subsp. IIIa no. SZ 203/92	53:z_4,z_{23}:−	+	Blue	Blue
Subsp. IIIa no. SZ 481/92	41:z_4,z_{23}:−	+	Blue	Blue
Subsp. IIIb no. 909	6,14:z_{10}:z	+++	Blue	Blue-violet
Subsp. IIIb no. 1127	38:1,v:z_{35}	+	Blue	Blue
Subsp. IIIb no. 1128	38:1,v:z_{50}:z_{54}	+++	Blue	None
Subsp. IIIb no. 1131	38:r:z	+++	Blue-violet	Blue-violet
Subsp. IIIb no. 1190	41:k:−	+	Blue	Blue
Subsp. IV no. 765	11:g,z_{51}:−	−	Beige	Orange, light edge
Subsp. IV no. 787	11:z_4,z_{23}:−	−	Colorless	Dark beige, light edge
Subsp. IV no. 957	16:z_4,z_{32}:−	−	Beige	Red, light edge
Subsp. IV no. 960	16:z_4,z_{32}:−	−	Beige	Dark beige, light edge; two colonies red, light edge
Subsp. V no. 1373	60:z_{41}:−	+	Blue	Blue
Subsp. V no. 1397	66:z_{41}:−	+++	Blue	Blue
Subsp. V no. 1398	66:z_{35}:−	++	Blue	Blue
Subsp. V no. 1399	66:z_{65}:−	++	Blue	Blue
Subsp. VI no. 864	6,14,25:a:e,n,x	++	Blue	Red-violet, light edge
Subsp. VI no. 751	11:b:e,n,x	−	Colorless	Red, light edge

1,663 strains were determined by the German National Reference Center for Salmonellosis in 1992. Of these strains, 1,637 (98.4%) belonged to subspecies I. The remainder consisted of seven strains of subspecies II, three of subspecies IIIa, seven of subspecies IIIb, and nine of subspecies IV (6). In an earlier study Pietzsch (7) reported on 12,886 *Salmonella* isolates recovered from various animals and foods in 1981. Within the total were 9 strains of subspecies II, 7 of subspecies IIIa (monophasic *S. arizonae* serovars), 22 of subspecies IIIb (biphasic *S. arizonae* serovars), and 5 of subspecies IV. No strains of subspecies V and VI were present. A total of 140 isolates belonged to the 43 serovars of subspecies II, IIIa, IIIb, and IV that were identified. Seventy-one isolates of *S. arizonae* 18:z_4,z_{32}:− were obtained from turkeys, and six isolates of *S. arizonae* 61:k:1,5,7 were obtained from sheep. Forty-five of the isolates belonging to subspecies II and IV came from reptiles and amphibians. The recently typed *Salmonella* strains of subspecies II to V were isolated from different animals and spices and from humans (Table 2). Most (53.4%) strains of *Salmonella* subspecies II to IV were isolated from humans in Germany from 1977 to 1992 (4). Of these strains, 81.9% caused diarrhea, gastroenteritis, and enteritis; 5.1% were isolated from patients with extraintestinal infections; and 13% were isolated from patients with latent infections.

Little is known about the epidemiology of the Arizona subgroup. Strains may be isolated from a wide variety of nonhuman and human sources, sometimes from extraintestinal sites. Some serovars of the Arizona subgroup (subspecies IIIa and IIIb) differ in their invasivity (9). This study has shown that when working with Rambach agar, the user must submit not only red colonies but also blue-green colonies for further examination. Although *Salmonella* subspecies II to VI are rare, the possibility of their presence in samples taken from sheep, turkeys, and cold-blooded animals cannot be excluded even though red colonies are not seen on Rambach agar.

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TABLE 2. Examination of *Salmonella* subspecies II to V from different origins isolated in 1993 on Rambach agar and by β-D-galactosidase (ONPG) reaction

Subspecies and strain	Antigen formula	Origin	ONPG test	Color on Rambach agar (single colonies)
Subsp. II no. SZ 936/93	47:a:1,5	Turtle	−	Red/crimson
Subsp. II no. SZ 1104	47:a:1,5	Turtle	−	Red/crimson
Subsp. II no. SZ 1131	47:a:1,5	Turtle	−	Red/crimson
Subsp. II no. SZ 1416	4:b:−	Human	−	Red/crimson
Subsp. II no. SZ 1533	9:m,t:1,5	Spice	−	Red/crimson
Subsp. II no. SZ 1534	35:z_{29}:−	Spice	−	Red/crimson
Subsp. II no. SZ 1574	60:z_{29}:e,n,x	Paprika	−	Red/crimson
Subsp. II no. SZ 1680	40:g,t:z_{39}	Snake	−	Red/crimson
Subsp. II no. SZ 1704	9:m,t:−	Spice	−	Red/crimson
Subsp. II no. SZ 1711	4:b:−	Poultry	−	Red/crimson
Subsp. II no. SZ 1712	4:b:−	Poultry	−	Red/crimson
Subsp. II no. SZ 1723	38:d:1,5	Spice	−	Red/crimson
Subsp. II no. SZ 1892	6.7:g,m,t:−	Paprika	−	Red/crimson
Subsp. IIIa no. SZ 99	41:z_4,z_{23}:−	Saurian (Agame)	+	Blue-violet
Subsp. IIIa no. SZ 143	41:z_4,z_{23}:−	Snake (boa constrictor)	+	Blue
Subsp. IIIa no. SZ 478	18:z_4,z_{32}:−	Turkey	+	Blue-violet
Subsp. IIIa no. SZ 830	53:z_4,z_{23}:−	Snake	+	Blue-violet
Subsp. IIIa no. SZ 1251	53:z_4,z_{23}:−	Viper	+	Blue-violet
Subsp. IIIb no. SZ 96	61:k:1,5	Fetus (sheep)	+	Blue
Subsp. IIIb no. SZ 144	35:l,v:z_{35}	Snake (boa constrictor)	+	Blue-violet
Subsp. IIIb no. SZ 220/93	48:l,v:1,5	Snake (boa constrictor)	+	Blue-violet
Subsp. IIIb no. SZ 668	61:l,v:z_{35}	Human	+	Blue-violet
Subsp. IIIb no. SZ 827	14:z_{10}:z	Viper	+	Blue-violet
Subsp. IIIb no. SZ 828	14:z_{10}:z	Viper	+	Blue-violet
Subsp. IIIb no. SZ 829	14:z_{10}:z	Viper	+	Blue-violet
Subsp. IIIb no. SZ 1495	61:−:1,5	Sheep	+	Blue
Subsp. IIIb no. SZ 1496	38:k:−	Viper	+	Blue-violet
Subsp. IIIb no. SZ 1497	38:k:−	Viper	+	Blue-violet
Subsp. IV no. SZ 456	16:z_4,z_{32}:−	Snake (boa constrictor)	−	Beige
Subsp. IV no. SZ 457	16:z_4,z_{32}:−	Green iguana	−	Light red
Subsp. IV no. SZ 852	50:z_4,z_{32}:−	Human	−	Beige
Subsp. IV no. SZ 916	16:z_4,z_{32}:−	Iguana	−	Light-red
Subsp. IV no. SZ 1442	50:z_4,z_{32}:−	Human	−	Beige
Subsp. IV no. SZ 1464	6,7:z_{36}:−	Human	−	Beige
Subsp. V no. SZ 1692	66:z_{35}:−	Paprika	+	Blue

Vorkommen von Salmonellen der Subspezies II–V in Deutschland, abstr. 109, 45. Tagung Dtsch. Ges. Hyg. Mikrobiol.

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