JOURNAL OF CLINICAL MICROBIOLOGY, Nov. 2002, p. 3913–3916 Vol. 40, No. 11
0095-1137/02/$04.00+0 DOI: 10.1128/JCM.40.11.3913–3916.2002

Alafosfalin as a Selective Agent for Isolation of *Salmonella* from Clinical Samples

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Received 18 April 2002/Returned for modification 27 June 2002/Accepted 31 July 2002

The selectivity of a range of culture media for the detection of *Salmonella* was assessed using 435 strains of gram-negative bacteria. These media showed limited ability to inhibit non-*Salmonella* strains found in stool samples. We report the evaluation of alafosfalin as a selective agent for isolation of *Salmonella* from stool samples. Susceptibility studies with this agent showed that non-*typhi Salmonella* strains were relatively resistant (mean MIC, 10.2 mg/liter) compared to many coliforms including *Escherichia coli* (mean MIC, 0.7 mg/liter). A chromogenic medium, ABC medium, was modified to incorporate alafosfalin and was compared with standard ABC medium and Hektoen enteric agar for the isolation of *Salmonella* from 1,000 stool samples. On direct culture, modified ABC medium showed higher recovery of *Salmonella* (53.6%) compared with either ABC medium (35.7%) or Hektoen enteric agar (48.2%). We conclude that alafosfalin is a useful selective agent for the isolation of *Salmonella* from stool samples.

There is a wide range of culture media available for the isolation of *Salmonella.* Traditional media such as deoxycholate citrate agar, Hektoen enteric (HE) agar, and xylose lysine decarboxylase agar (XLD) rely mainly on the detection of hydrogen sulfide production and/or nonfermentation of lactose by *Salmonella* (12). Although these media may be highly effective, they are nonspecific, and a number of species may produce colonies resembling *Salmonella* (4, 12).

The introduction of chromogenic media which employ enzyme substrates has allowed an increased specificity in the detection of *Salmonella* (7). Rambach agar (10) and *Salmonella* detection and identification medium (SM-ID) medium (M. C. Poupart, M. Mounier, F. Denis, J. Sirot, C. Couturier, and F. Villeval, Abstr. 5th Eur. Congr. Clin. Microbiol. Infect. Dis, abstr. 1254, 1991) were the first media of this type. Rambach agar uses a chromogenic substrate for β-galactosidase (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside [X-Gal]) in conjunction with propylene glycol, which is fermented by *Salmonella* spp. to generate acid (10). SM-ID medium is based on similar principles incorporating X-Gal and glucuronic acid (Poupart et al., Abstr. 5th Eur. Congr. Clin. Microbiol. Infect. Dis.). ABC medium, more recently commercialized, contains a combination of two chromogenic substrates, and *Salmonella* is detected on the basis of its production of α-galactosidase (9). In recent years a range of media have been manufactured which rely on the detection of esterase activity by *Salmonella* using chromogenic substrates (2, 5, 6, 8).

Despite over a decade of development in the field of chromogenic media for *Salmonella,* there is much evidence to suggest that traditional media may offer a superior recovery rate for the direct isolation of *Salmonella* from stool samples (3, 4, 7; J. D. Perry, G. Riley, and F. K. Gould, Abstr. 101st Gen. Meet. Am. Soc. Microbiol. 2001, abstr. C169, 2001; C. Roure, J. M. Perez, P. Cavalli, and A. M. Freydière, Abstr. 101st Gen. Meet. Am. Soc. Microbiol. 2001, abstr. C170 p. 188, 2001). In this study we assess the selectivity of a selection of both chromogenic and conventional agars for *Salmonella* and investigate a novel approach to the selective isolation of *Salmonella* using a "suicide substrate," alafosfalin (1).

Alafosfalin (L-alanyl-1-aminoethylphosphonic acid) is taken up by bacteria at different rates via a stereospecific permease and may be cleaved intracellularly by an aminopeptidase to yield fosfalin (1-aminoethylphosphonic acid) (1). This released compound interferes with bacterial metabolism via interaction with the alanine racemase enzyme responsible for generating D-alanine (11). This interaction may result in growth inhibition.

In this study, the activity of alafosfalin was assessed against a wide range of gram-negative bacteria. Alafosfalin was then incorporated into a modified version of ABC medium. The sensitivity and specificity of this medium were assessed for the isolation of *Salmonella* from stool samples in comparison with HE agar and ABC medium.

MATERIALS AND METHODS

HE agar, SM-ID medium, and selenite broth were purchased from bioMérieux UK Ltd., Basingstoke, United Kingdom, as ready-to-use media. CHROMagar *Salmonella* (CAS) and Rambach agar (RAM) were purchased as dehydrated media from M-Tech, Warrington, United Kingdom. ABC medium, XLD agar, and nutrient agar were purchased as dehydrated media from Bioconnections Ltd., Leeds, United Kingdom. All dehydrated media were prepared in strict accordance with the manufacturers' instructions.

A modified version of ABC medium was prepared for incorporation of alafosfalin since the activity of this agent required a defined medium free of peptones. For modified ABC medium, amino acids, nucleotide bases, and alafosfalin were obtained from Sigma Chemical Co., Poole, United Kingdom. 5-Bromo-4-chloro-3-indolyl-α-D-galactoside (X-alpha-Gal) and isopropyl-β-D-thiogalactoside (IPTG) were obtained from Glycosynth, Warrington, United Kingdom. 3,4-

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TABLE 1. Growth of gram-negative bacteria on various *Salmonella* agars

Strain	No. tested	% of strains showing growth on: ABC	MABC	CAS	RAM	SMID	HE	XLD
Total non-*Salmonellae*	372	87.9	54.6	77.4	79.8	96.5	70.4	90.9
Total β-galactosidase producers	235	95.7	46	93.6	98.7	99.6	73.6	89.8
Total *E. coli*	108	96.3	10.2	95.4	98.1	100	45.4	78.7
Salmonella spp. (serovar non-Typhi)	61	100	100	100	100	100	100	100
S. enterica serovar Typhi	2	100	0	100	100	100	100	100

Cyclohexenoesculetin-β-D-galactoside (CHE-Gal) was purchased from Lab M, Bury, United Kingdom. All other constituents were obtained from BDH Chemicals Ltd, Poole, United Kingdom.

For preparation of modified ABC medium, the following ingredients were weighed out and added to 1 liter of water: bacteriological agar (10 g), L-arginine (0.1 g), L-aspartic acid (0.1 g), L-cysteine (0.005 g), glycine (0.1 g), L-histidine (0.1 g), L-isoleucine (0.1 g), L-leucine (0.1 g), L-lysine (0.1 g), L-methionine (0.005 g), L-phenylalanine (0.1 g), L-proline (0.1 g), L-serine (0.1 g), L-threonine (0.1 g), L-tryptophan (0.1 g), L-tyrosine (0.1 g), L-valine (0.1 g), guanine (0.01 g), uracil (0.01 g), cytosine (0.01 g), adenine (0.01 g), sodium citrate (6.5 g), magnesium sulfate (0.1 g), ammonium sulfate (1 g), yeast extract (0.1 g), dipotassium hydrogen phosphate (7 g), and potassium dihydrogen phosphate (2 g). The chromogenic mix consisted of ferric ammonium citrate (0.5 g), X-alpha-Gal (0.08 g), CHE-Gal (0.3 g), and IPTG (0.03 g). The above mixture was sterilized at 116°C for 10 min, cooled to below 100°C, and supplemented with sodium deoxycholate (0.5 g).

Alafosfalin was dissolved in water, filter sterilized, and double diluted in sterile water to produce a concentration range of 640 to 2.5 mg/liter. It was then added to the above medium at 50°C to produce a final concentration range in agar of 32 to 0.125 mg/liter. A total of 25 plates were prepared at each concentration.

A collection of 435 gram-negative bacteria were used in this study. Each strain had been previously isolated from pathological samples and identified using either API 20E or API 20 NE (bioMerieux UK Ltd., Basingstoke, United Kingdom). All strains of *Salmonella* had also been serotyped by the Central Public Health Laboratory, London, United Kingdom. Commonly occurring serotypes were also phage typed.

Before being tested, strains were retrieved from storage in glycerol at −20°C and subcultured onto nutrient agar. Each strain was emulsified in 1 ml of physiological saline (0.85%) to a suspension equivalent to a McFarland 0.5 standard using a densitometer (approximately 1.5×10^8 CFU/ml). Using a multipoint inoculator (Mast Laboratories, Bootle, United Kingdom), 1 μl of each suspension was inoculated onto HE, SM-ID, CAS, RAM, ABC, XLD, and nutrient agar. All strains were also inoculated onto modified ABC medium containing the various concentrations of alafosfalin as well as modified ABC without alafosfalin. The collection comprised *Acinetobacter* spp. ($n = 49$), *Aeromonas caviae* ($n = 5$), *Aeromonas hydrophila* ($n = 3$), *Citrobacter diversus* ($n = 9$), *Citrobacter freundii* ($n = 16$), *Citrobacter koseri* ($n = 3$), *Enterobacter aerogenes* ($n = 10$), *Enterobacter agglomerans* ($n = 1$), *Enterobacter cloacae* ($n = 20$). *Escherichia coli* ($n = 108$), *Escherichia hermanii* ($n = 1$), *Hafnia alvei* ($n = 10$), *Klebsiella oxytoca* ($n = 13$), *Klebsiella pneumoniae* ($n = 22$), *Kluyvera* sp. ($n = 1$), *Morganella morganii* ($n = 11$), *Proteus mirabilis* ($n = 14$), *Proteus penneri* ($n = 1$), *Proteus vulgaris* ($n = 6$), *Providencia alcalifaciens* ($n = 3$), *Providencia rettgeri* ($n = 2$), *Providencia stuartii* ($n = 10$), *Pseudomonas aeruginosa* ($n = 7$), *Salmonella* spp. ($n = 63$), *Serratia liquefaciens* ($n = 6$), *Serratia marcescens* ($n = 7$), *Shigella boydii* ($n = 2$), *Shigella dysenteriae* ($n = 3$), *Shigella flexneri* ($n = 2$), *Shigella sonnei* ($n = 10$), *Yersinia enterocolitica* ($n = 14$) and *Yersinia pseudotuberculosis* ($n = 3$). All cultures were incubated for 18 h at 37°C, and the colony characteristics of each strain were recorded. The MIC of alafosfalin was recorded as the lowest concentration which prevented visible growth on modified ABC medium.

A total of 1,000 fresh stool samples were processed within the four different participating laboratories on the day they were received in the laboratory. A 1-g or 1-ml sample of stool was suspended in 10 ml of physiological saline (0.85 %) and emulsified to produce an even suspension. A 1-g or 1-ml sample of stool was simultaneously inoculated into 10 ml of selenite broth. A 10-μl aliquot of the fecal suspension was then cultured onto ABC medium, modified ABC medium (containing 1 mg of alafosfalin per liter), and HE agar. All plates were examined daily for the presence of *Salmonella* and incubated for up to 48 h at 37°C. After overnight incubation at 37°C, the selenite broth was subcultured onto the same three media in identical fashion. These plates were also incubated for up to 48 h at 37°C.

RESULTS AND DISCUSSION

All *Enterobacteriaceae* strains grew on nutrient agar and on modified ABC medium without alafosfalin. Alafosfalin showed no detectable activity (MIC, >32 mg/liter) against any strains of *Acinetobacter, Pseudomonas, Proteus, Providencia,* or *Morganella.* Alafosfalin inhibited the growth of most strains of *E. coli* (mean MIC, 0.7 mg/liter; MIC range, 0.125 to >32 mg/liter) and *Shigella* spp. (mean MIC, 0.25 mg/liter; MIC range, 0.125 to 0.5 mg/liter). Moderate activity was shown against *Klebsiella* spp. (mean MIC, 5.9 mg/liter; MIC range, 1 to 16 mg/liter), *Serratia* spp. (mean MIC, 3.2 mg/liter; MIC range, 1 to 8 mg/liter), *Enterobacter* spp. (mean MIC, 6.4 g/liter; MIC range, 0.125 to 32 mg/liter), *Citrobacter* spp. (mean MIC, 2.8 mg/liter; MIC range, 0.25 to 16 mg/liter), *Aeromonas* spp. (mean MIC, 2 mg/liter; MIC range, 0.125 to 4 mg/liter), *Yersinia* spp. (mean MIC, 16.6 mg/liter, MIC range, 4 to >32 mg/liter), and *Hafnia alvei* (mean MIC, 2.8 mg/liter; MIC range, 1 to 4 mg/liter).

Of 63 strains of *Salmonella* (including 40 distinct serotypes), most were moderately resistant to alafosfalin (mean MIC, 10.2 mg/liter; MIC range, 2 to 32 mg/liter), except for two strains of *Salmonella enterica* serovar Typhi, which were highly sensitive (MIC, 0.25 mg/liter). Of the 61 non-Typhi *Salmonella* strains, all grew well on modified ABC medium containing 1 mg of alafosfalin per liter and demonstrated a typical green coloration due to production of α-galactosidase. This formulation containing 1 mg of alafosfalin per liter was compared in detail with the other *Salmonella* agars tested; for the remainder of this report, it is referred to as modified ABC medium (MABC).

All of the *Salmonella* agars tested were highly effective in growing pure strains of *Salmonella* (Table 1); however, their ability to inhibit other gram-negative bacteria was less effective. Of the commercially available agars, HE agar was the best at inhibiting non-*Salmonella* strains, even though 70.4% of strains were allowed to grow. Almost half of the 372 non-*Salmonella* strains tested did not grow on MABC medium, and, significantly, only 10.2% of *E. coli* strains showed any growth on this medium.

After enrichment, a total of 56 *Salmonella* strains were isolated from 1,000 stool samples (Table 2). These strains comprised 11 distinct serotypes including serovars Enteritidis, Hadar, Javiana, Montevideo, Ohio, Panama, Thompson, Typhi, Typhimurium, Vilvorde, Virchow, and unnamed *Salmonella* serotypes (*Salmonella* group B, *Salmonella* group G1, and *Salmonella* group OMC).

One strain of serovar Enteritidis and one strain of serovar Vilvorde were not recovered using HE agar before or after

TABLE 2. Comparative evaluation of three media for recovery of *Salmonella* from clinical samples at four different laboratories

Laboratory location	No. of samples	No. of *Salmonella* strains isolated after 24 h of incubation on:						
		Any media	HE medium		ABC medium		MABC medium	
			BE[a]	AE[a]	BE	AE	BE	AE
Newcastle upon Tyne, United Kingdom	797	33	18	32	17	33	25	33
Lyon, France	34	0	0	0	0	0	0	0
Point-à-Pitre, Guadeloupe	75	3	0	3	0	3	0	3
Burkina Faso	94	20	9	19	3[b]	10[b]	5[b]	8[b]
Total no.	1,000	56	27	54	20	46	30	44

[a] BE, before enrichment in Selenite broth; AE, after enrichment in Selenite broth.
[b] Nine non-Typhi *Salmonella* strains including six serovar Typhimurium strains, one serovar Enteritidis strain, one serovar Virchow strain, and one *Salmonella* group B strain were not detected using ABC and MABC media, since they did not produce α-galactosidase. Two strains of serovar Typhi failed to grow on MABC medium.

enrichment. Six strains of serovar Typhimurium, one strain of serovar Enteritidis, one strain of serovar Virchow, and one strain of *Salmonella* group B were not recovered using ABC and MABC media, and two strains of serovar Typhi failed to grow on MABC medium. The nine non-Typhi *Salmonella* strains which were not detected on ABC and MABC media were all isolated in Burkina Faso from different patients. A retrospective analysis of the culture plates showed that all of these *Salmonella* strains had grown on both media on direct culture but had failed to generate green colonies. When subcultured onto ABC medium, these colonies remained white due to lack of α-galactosidase. This factor accounted for the apparent differences between the different media postenrichment. The lack of α-galactosidase production by these strains was confirmed at the Freeman Hospital Microbiology Laboratory using the fluorogenic substrate 4-methylumbelliferyl-α-D-galactoside.

On primary isolation, there was little difference between the performance of ABC medium and HE agar for recovery of *Salmonella,* except in the Burkina Faso laboratory for reasons explained above. On direct culture, MABC medium showed a higher recovery of *Salmonella* (53.6%) than did either ABC medium (35.7%) or HE agar (48.2%), even though at least nine strains of *Salmonella* remained undetected on MABC medium due to lack of α-galactosidase production. It is likely, therefore, that these numbers substantially underestimate the utility of alafosfalin as a selective agent.

HE agar was far less specific than the chromogenic agars, and a total of 313 non-*Salmonella* strains on HE agar required further identification to exclude *Salmonella.* This compared with 21 non-*Salmonella* strains generating green colonies on ABC medium and 57 false-positive strains on MABC medium. All of the media showed an increase in the number of false-positive colonies after 48 h of incubation (data not shown); however, HE agar remained by far the least specific in all laboratories.

The lack of selectivity of chromogenic agars is well recognized, and this has led to the suggestion that they should not be recommended for primary isolation of *Salmonella* from stool samples (3, 4). In this study, all of the commercially available chromogenic media readily supported the growth of more than 95% of *E. coli* strains tested and the vast majority of strains produced intensely coloured growth due to β-galactosidase production. These data may provide an explanation for the limited ability of such media to recover *Salmonella* on primary culture. Modified ABC medium, containing 1 mg of alafosfalin per liter, had a high impact on the selective inhibition of non-*Salmonella* strains, and its performance was superior in this respect to that of any of the commercially available agars tested. The recovery of *Salmonella* from stool samples was also better on MABC than on HE agar, a medium which is often used as a "gold standard" in other studies (3, 5, 7; Roure et al., Abstr. 101st Gen. Meet. ASM 2001).

The finding that 9 of 20 strains of *Salmonella* isolated in the Burkina Faso laboratory failed to produce green colonies on either ABC medium or MABC medium was notable. Strains which do not produce α-galactosidase are rare but have been previously recognized (9); however, the high prevalence of these strains (45%) in this geographical location was highly unexpected and places limitations on the use of media which rely on the detection of α-galactosidase production.

In conclusion, this report has highlighted the utility of a novel selective agent, alafosfalin, for the isolation of non-Typhi *Salmonella* strains. Alafosfalin is inexpensive, highly stable, and readily soluble in water. This approach, using a "suicide substrate" is substantially different from those traditionally used for selectivity in that activity of specific enzymes is required for inhibition. It is possible that such suicide substrates could be custom designed as selective agents for the isolation of a wide range of other pathogens in the future.

ACKNOWLEDGMENTS

We thank the Regional Public Health Laboratory, Newcastle upon Tyne, United Kingdom, for providing some of the stool samples and the Central Public Health Laboratory, London, United Kingdom, for performing serotyping and phage typing of *Salmonella* strains.

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