NOTES

Comparison of Preservation Methods for Enterotoxigenic *Escherichia coli* Producing Heat-Labile Enterotoxin

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Ten strains of enterotoxigenic *Escherichia coli* producing heat-labile enterotoxin (LT) were preserved under 12 different conditions. After 1 month, 9 months, and 3 years of preservation, the cultures were recovered and examined for LT production. Preservation of the cultures on Dorset Egg Medium at 4°C and preservation by freezing the cell suspensions in tryptic soy broth with 20% glycerol were found to be suitable preservation methods; all strains were alive for 3 years and had a minimum loss of LT production.

Production of heat-labile enterotoxin (LT) or heat-stable enterotoxin (ST) or both is the hallmark of enterotoxigenic *Escherichia coli* (ETEC). However, it is known that ETEC strains can frequently lose their ability to produce the enterotoxins (4, 5). This is primarily because the genes encoding LT and ST are present on so-called Ent plasmids, which are not stably maintained in some strains (1, 3). The stability of Ent plasmids may be influenced by various factors such as temperature or medium. Our experience has been that ETEC strains often lose their enterotoxigenicity during preservation of the cultures. Although various methods to detect LT and ST produced by ETEC strains have been devised (10, 11), circumstances may not allow examination of enterotoxin production upon initial isolation of ETEC candidates. Preservation of strains which have been identified as ETEC is also necessary. Therefore, it is considered important to determine the stability of the enterotoxigenicity of ETEC under various preservation conditions and to use the preservation method that not only supports survival of the organism but also allows a minimum loss of enterotoxigenicity. Some investigators have reported the stability of LT and ST production by ETEC strains after transfers of a stab culture (4) and the stability of LT production after storage of the culture at -70°C (2), but no systematic study comparing preservation methods for stability of the enterotoxigenicity of ETEC has been done to our knowledge. In this study, using the ability to produce LT as an indicator of the presence of Ent plasmids, we preserved LT-producing ETEC strains under various conditions for up to 3 years and examined LT production by the cultures at intervals to find the preservation method suitable for ETEC strains.

ETEC strains used in this study had originally been isolated from different travelers with diarrhea at the quarantine station of Osaka International Airport over a period of 2

years. The isolates were characterized for production of LT by Biken test (6, 7) and for that of ST by a suckling mouse assay (2) upon isolation. An O:K serotype was determined for some strains by Teizo Tsukamoto of the Osaka Prefectural Institute of Public Health. The isolates were grown at 37°C and maintained on Dorset Egg Medium (a slant medium composed of chicken egg and 0.9% NaCl solution in a 4:1 ratio; Nissui Pharmaceutical Co., Tokyo, Japan). Ten LT-producing strains listed in Table 1 were randomly selected from a collection of the above isolates for this study. Immediately before use, LT production by the test strains was confirmed, and these strains were preserved by various methods listed in Table 2. The commercially available media chosen for preservation in stab culture included the following: Nutrient Agar (Difco) prepared as a slant medium (NAs); Dorset Egg Medium, a slant medium (DEs); Nutrient Broth (Difco) with 0.7% Bacto Agar (Difco) (soft nutrient agar [sNA]); heart infusion broth (Difco) with 0.7% Bacto Agar (soft heart infusion agar [sHI]). The test organisms were inoculated on or in these media and stored at room temperature or in a cold (4°C) room (designated by “-R” or “-C,” respectively, after the abbreviation of the growth medium). The test organisms were also grown on heart

TABLE 1. Bacterial strains

Strain designation	Original strain no.	Production ^a of:		Serotype
		LT	ST	
1	8-307-1	+	-	O1:K51
2	8-414-1	+	-	O126:K71
3	8-1130-5	+	-	Unknown
4	324-4	+	+	O6:K15
5	8-1138-1	+	-	Unknown
6	8-393-1	+	+	O6:K15
7	8-824-2	+	-	O6:K15
8	8-352-1	+	+	O6:K15
9	8-323-1	+	+	O6:K15
10	8-306-3	+	+	O6:K15

^a +, positive; -, negative.

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TABLE 2. Effect of storage conditions on LT production in ETEC

Strain	Period of storage	% Loss of LT production under various storage conditions ^a											
		NAs-R	NAs-C	DEs-R	DEs-C	sNA-R	sNA-C	sHI-R	sHI-C	SM-LY	HIG-D	SMI-F	TSG-F
1	1 mo	0	0	0	0	0	0	20	0	0	0	0	0
	9 mo	0	0	0	0	0	0	0	0	0	0	0	0
	3 yr	0	0	0	0	0	0	0	0	0	0	0	0
2	1 mo	0	0	0	0	0	0	0	0	0	0	0	0
	9 mo	0	0	0	0	0	0	0	0	0	0	0	0
	3 yr	0	0	0	0	0	0	0	NG	0	0	0	0
3	1 mo	0	0	0	0	0	0	0	0	0	0	0	0
	9 mo	0	0	0	0	0	0	0	0	0	0	0	0
	3 yr	0	0	0	0	0	0	0	NG	0	0	0	0
4	1 mo	0	0	0	0	0	40	0	0	0	0	0	0
	9 mo	0	20	20	0	0	0	0	0	0	0	0	0
	3 yr	40	0	0	0	0	0	0	NG	0	0	0	0
5	1 mo	0	0	0	0	20	0	0	0	0	0	0	0
	9 mo	0	0	0	0	0	0	0	0	0	100	0	0
	3 yr	100	0	40	40	80	80	NG	NG	100	60	0	0
6	1 mo	0	0	0	0	0	0	0	0	0	0	0	0
	9 mo	40	0	0	0	0	0	0	0	0	0	0	0
	3 yr	20	NG	0	0	0	0	0	NG	0	20	NG	0
7	1 mo	0	0	0	0	0	0	0	0	0	0	0	0
	9 mo	0	0	0	0	0	0	80	0	0	0	0	0
	3 yr	60	NG	100	0	0	0	NG	NG	0	0	NG	0
8	1 mo	40	0	0	0	60	0	0	0	0	0	0	0
	9 mo	0	NG	40	0	0	NG	20	NG	0	0	0	0
	3 yr	20	NG	0	20	20	NG	100	NG	40	80	0	0
9	1 mo	0	0	0	0	20	50	0	0	0	0	20	0
	9 mo	0	0	0	0	100	0	40	0	0	20	NG	0
	3 yr	60	NG	100	0	100	NG	100	NG	0	0	NG	0
10	1 mo	80	100	80	60	60	50	100	80	100	100	80	60
	9 mo	100	NG	100	60	100	20	100	NG	80	100	80	80
	3 yr	100	NG	100	80	100	NG	NG	NG	100	100	NG	100

^a Five colonies per sample were examined as described in the text. The percentage of colonies which lost the ability to produce LT (see text for calculation) are shown. NG, no growth was observed when the stock culture was streaked out after storage. Conditions: NAs-R, nutrient agar slant at room temperature; NAs-C, nutrient agar slant at 4°C; DEs-R, Dorset Egg Medium slant at room temperature; DEs-C, Dorset Egg Medium slant at 4°C; sNA-R, soft nutrient agar at room temperature; sNA-C, soft nutrient agar at 4°C; sHI-R, soft heart infusion agar at room temperature; sHI-C, soft heart infusion agar at 4°C; SM-LY, lyophilized in 10% skim milk solution; HIG-D, suspended in heart infusion broth-gelatin solution and dried on paper disks; SMI-F, frozen (−80°C) in 10% skim milk–10% inositol solution; TSG-F, frozen (−80°C) in tryptic soy broth with 20% glycerol.

infusion agar (Difco), harvested, lyophilized in 10% Bacto Skim Milk (Difco) solution, and stored at −20°C (SM-LY). The harvested cells were suspended in a mixture of heart infusion broth and 20% gelatin solution (1:1), absorbed onto paper disks, dried, and stored at room temperature as described by Stamp (12) (HIG-D). The harvested cells were also suspended in 10% Bacto Skim Milk solution with 10% inositol (SMI) or in tryptic soy broth (Difco) with 20% glycerol (TSG) and stored in a freezer maintained at −80°C (designated by “-F” after the abbreviation of the suspending medium).

After 1 month, 9 months, and 3 years, the preserved cultures were streaked out onto heart infusion agar plates. Five isolated colonies per test specimen were chosen at random and tested for production of LT by the Biken test. Percent loss of LT production was then calculated: number of LT-negative clones divided by number of examined clones and multiplied by 100 (Table 2).

Previous workers noted strain-to-strain variation in the stability of LT production by ETEC strains after storage of stab (4) and frozen (5) cultures. Our results summarized in Table 2 confirmed the strain-to-strain variation to occur under various preservation conditions; e.g., strain 1 fully maintained the ability to produce LT after 3-year storage, while the majority of the subclones of strain 10 isolated after 1-month storage did not produce LT. To confirm that the loss of LT production was principally due to segregation of the Ent plasmid carrying the LT gene, subclones of strain 10, an LT- and ST-positive strain (Table 1), isolated after 3-year preservation on DEs were examined for absence of the LT gene and for loss of ability to produce ST. Presence or absence of the LT gene was determined by the DNA colony hybridization method, as described previously (9). The LT gene probe originally described by Moseley et al. (8), a 0.8-MDa *HincII* DNA fragment encoding predominantly the A subunit of LT, was obtained through James B. Kaper of

University of Maryland and was used as the hybridization probe. Of the nine LT-negative clones (five from DEs-R and four from DEs-C), eight clones neither had the LT gene nor produced ST, indicating loss of the Ent plasmid. The one remaining clone did have the LT gene and produced ST, suggesting that mutation affecting the level of LT production or the antigenicity of LT had occurred. A study by Evans et al. (4) suggested an association of LT production stability with ETEC strains serotype. Due to the limited number of strains used in this study, additional evidence in support of their observation cannot be provided.

Medium or method of preservation apparently did not have a significant influence on the stability of LT production by strains which were very stable (strains 1 to 3) or extremely unstable (strain 10) for LT production. The results obtained with strains 4 to 9, with intermediate stability of LT production, indicated that some preservation media or methods are superior to others for maintaining ETEC strains. Of the culture media used for the subculturing method (NAs, DEs, sNA, and sHI), DEs appears to be the medium of choice. On the whole, this medium gave better results than the other nutrient- or heart-infusion-based media in terms of stability of LT production. DEs combined with a low preservation temperature (DEs-C) gave slightly better stability than DEs combined with preservation at room temperature (DEs-R), and both methods using DEs kept all 10 test strains alive for 3 years, whereas the low preservation temperature used in combination with the other media (NAs-C, sNA-C, and sHI-C) induced death of three to nine test strains in 3 years.

Of the methods which do not require periodic transfer (SM-LY, HIG-D, SMI-F, and TSG-F), freezing in TSG (TSG-F) gave the best results. All test strains were kept alive for at least 3 years, and no loss of ability to produce LT was observed with the test strains other than strain 10 by the TSG-F method. However, freezing in SMI (SMI-F) caused death of four strains in 3 years, and neither the lyophilization (SM-LY) nor the drying (HIG-D) method provided stability of LT production as equal to that provided by the TSG-F method.

DEs-C and the TSG-F method gave better results than any of the other media or methods even when strain 10 was tested. Accordingly, we recommend these two conditions for maintaining ETEC strains. DEs-R seems to be the condition of choice when a refrigerator or freezer is not available, and DEs may also be used for transport of ETEC isolates.

This work was supported by a grant-in-aid for scientific research from the Ministry of Education, Science and Culture of Japan.

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