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Evaluation of Two *Salmonella typhi* Strains with Reduced Virulence for Use in Teaching and Proficiency Testing

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A total of 21 cases of laboratory-acquired typhoid fever associated with teaching and proficiency tests occurred in the United States during a 33-month period, prompting a search for less virulent strains of *S. typhi* which would be suitable for teaching purposes. Two strains were evaluated which are reported to have reduced virulence for mice. Strain Ty21a is a genetically constructed mutant that lacks the enzyme UDP-glucose-4-epimerase. This strain has reduced virulence for humans if grown under special laboratory conditions (in the presence of 0.1% D-galactose) and has been evaluated as a candidate for use as a live, oral vaccine. Strain H901 was originally isolated in Russia in 1918. It has not been tested in humans, but its nonmotile variant, O901, has been found to be somewhat less virulent for humans; however, it can cause infection with doses of 10^7 organisms. In teaching exercises, all strains should be treated as though they are fully virulent. Ty21a and H901 were satisfactory, but not ideal, for teaching purposes. Biochemically, they could be identified by conventional tests and by commercially available diagnostic systems, although Ty21a was H_2S negative. Serologically, both strains posed problems. Both Ty21a and H901 were Vi antigen negative, and Ty21a was rough and grew poorly. Both strains were susceptible to antibiotics, including chloramphenicol, ampicillin, and trimethoprim-sulfamethoxazole. When Ty21a and H901 were mixed with *Escherichia coli* and plated, Hektoen and salmonella-shigella agars were most useful for their recovery. The appearance of Ty21a and H901 on differential plating media was typical, although Ty21a had smaller colonies. The plating efficiency on MacConkey agar for Ty21a was 0.6 compared with 1 for H901. Neither strain can be recommended unequivocally for teaching purposes; instead, the advantages and disadvantages of each must be considered. Both strains have been deposited in the American Type Culture Collection (Ty21a = ATCC 33459 = CDC 2861-79; H901 = ATCC 33458 = CDC 2862-79).

In early 1979, the Centers for Disease Control (CDC) learned of two cases of typhoid fever that were associated with a laboratory proficiency test. Since these cases and several other cases that were subsequently reported proved to be laboratory acquired, an extensive study was conducted covering the period from 1 January 1977 to 30 September 1979. A total of 24 cases of laboratory-acquired typhoid fever in the United States that occurred during this period were identified; 21 of these cases were associated with laboratory training, proficiency tests, or research (2). All of these cases were associated with strains of *Salmonella typhi* that had been originally isolated from typhoid cases. The risk of handling such strains is apparently greater than was supposed. To reduce the risk of laboratory-acquired typhoid, it seems advisable to use strains that are incapable of causing typhoid in humans.

Unfortunately, no strain of *S. typhi* is completely safe, but two strains, Ty21a and H901, have been reported to have reduced virulence for mice, and Ty21a has been reported to have reduced virulence for humans (4–7, 9, 10). Two factors have traditionally been considered important in virulence studies of *S. typhi*, the presence of Vi antigen and the ability to kill mice on intraperitoneal injection (7, 8). The 50% lethal dose in mice was greater than 10^8 organisms (suspended in saline) for these two strains compared with 10^7 organisms for more virulent strains (6, 10, 12). Ty21a is a genetically constructed mutant of Ty2, a highly virulent strain originally isolated in Russia in 1918 (7). Ty21a lacks the enzyme UDP-glucose-4-epimerase (EC

TABLE 1. *Salmonella typhi strains evaluated in this study.*

Original no.	ATCC No.	CDC No.	Description (reference)[a]
Ty 21a	33459	2861-79	Mutant of virulent strain Ty 2; lacks uridine 5'-diphosphate-glucose-4-epimerase; evaluated as live, oral vaccine (9, 10).
H 901	33458	2862-79	Originally isolated with TY 2; 0 901, nonmotile variant of H 901, may cause disease in humans with dose of 10^7 organisms. (5, 6, 7, 12)

[a]Both strains have reduced virulence for mice (6, 9).

5.1.3.2 [13]), and is referred to as the *gal* E mutant. This strain does not ferment galactose. It is a rough mutant that lyses when grown in glucose-free media containing galactose (9). This strain was less virulent for mice and has been evaluated in humans as a live, attenuated oral vaccine (10). When five to eight doses of vaccine, containing 3×10^{10} to 10×10^{10} viable organisms per dose (grown in the presence of galactose), were given orally to 155 men, no infection or significant side effects were noted (10). H901 was isolated at the same time as Ty2 (7) but, unlike Ty2, was reported less virulent for mice (6). Although H901 has not been tested in humans, a nonmotile variant of H901, O901, was used in human studies. When 20 volunteers ingested 10^7 O901 organisms, 6 (30%) of them became ill with typhoid, 30% had a milder infection, and 40% had no infection. When three Vi antigen-positive strains that are highly virulent for mice were tested in this same study (same dose) in 47 volunteers, 51% contracted a severe case of typhoid, 40% had a milder form, and only 9% had no infection (12).

The two strains described here were evaluated to determine whether they would be satisfactory for teaching purposes. These evaluations included biochemical tests, agglutination reactions in salmonella O, H, and Vi antisera, antibiograms, phage typing, identification by commercial diagnostic systems, growth and appearance on plating media, recovery from mixed cultures, and effects of lyophilization. No virulence tests were done in this study. The results of these tests and our recommendations are presented here. Ty21a and H901 have been deposited in the American Type Culture Collection (ATCC) for individuals or organizations wishing to use them (see Table 1).

MATERIALS AND METHODS

Bacterial strains. The two *S. typhi* strains were obtained as lyophilized cultures from L. S. Baron, Walter Reed Army Institute of Research, Washington, D.C. These strains are referred to as the test strains and are described in Table 1. They were grown on blood agar at 36°C and frozen in 10% skim milk at −70°C. Two typical strains of *S. typhi*, CDC 3198-74 and CDC 3219-74, were chosen from the stock cultures for study as controls. All the strains were also stored at room temperature on Pai medium (11). A strain of *Escherichia coli*, CDC E4115, was used in the mixed-culture study.

General. All experiments were done on all four strains. These strains were then freeze-dried by the Licensure and Proficiency Testing Division of CDC to determine how well they survived. Conventional biochemical tests, antibiograms, phage typing, and some serology were repeated after lyophilization. After these tests were completed and proved satisfactory, the two test strains were deposited in ATCC where they were also lyophilized. The lyophilized cultures from ATCC were then retested in the same way. Only the results obtained for the two test strains are reported (see Tables 2 to 7), since the control strains were typical and the reactions expected for *S. typhi* have been fully documented (11). All reactions before and after lyophilization were the same unless otherwise noted. All reactions were at 36 ± 1°C unless otherwise noted.

Media. Whenever possible, commercial dehydrated media were used. The media for biochemical tests, serology, antibiograms, and phage typing have been described (11). Blood agar refers to Trypticase soy agar with 5% defribrinated sheep blood (BBL Microbiology Systems, Cockeysville, Md.). The media used for lyophilization included Todd-Hewitt broth and polyvinylpyrrolidone-inositol-sodium glutamate medium (3). Three commercial diagnostic identification systems (kits) were used: Enterotube II, Roche Diagnostics, Div. Hoffman-LaRoche, Inc., Nutley, N.J.; Micro-ID, General Diagnostics, Warner-Lambert Co., Morris Plains, N.J.; and API 20E, Analytab Products, Plainview, N.Y.

Biochemical tests. Conventional biochemical tests were done on Ty21a and H901 (see Table 2). The methods used for these tests are those recommended by Edwards and Ewing, with some modifications (11). The three kits listed above (see Table 3) were also used for identification of the strains by the instructions of the manufacturers. These kit tests were not repeated after lyophilization.

Agglutination in salmonella antisera. Serology was done by the standard methods used in the Enteric Section at CDC (11) with antisera prepared at CDC. The somatic (O) antigens (see Table 4) were determined by slide agglutination on 24-h cultures suspended in 95% ethyl alcohol, heat killed at 60°C for 30 min, and suspended in phenolized saline (11). The flagellar

(H) antigen was detected by tube agglutination on 24-h broth (equal volumes of Trypticase soy and tryptose broths) cultures that had been formalinized for 6 h (11). Tests for the Vi antigen were done on 24-h cultures grown on blood agar and suspended in phenolized saline (11). These tests were repeated after lyophilization. In addition, commercial O group D, O polyvalent, and Vi antisera (Lee Laboratories, Grayson, Ga.) were used to test live and heated (100°C for 10 min) antigens grown on Trypticase soy agar. Tests with commercial antisera were not repeated after lyophilization.

Antibiograms. The standardized disk method was used (11). The antimicrobial agents and their concentrations are as follows: ampicillin, 10 μg; carbenicillin, 100 μg; cephalothin, 30 μg; chloramphenicol, 30 μg; colistin, 10 μg; gentamicin, 10 μg; kanamycin, 30 μg; nalidixic acid, 30 μg; penicillin, 10 U; streptomycin, 10 μg; tetracycline, 30 μg; and trimethoprim-sulfamethoxazole, 1.25 μg + 23.75 μg. The strains were grown in Trypticase soy broth for 24 h, diluted to the turbidity of the 0.5 McFarland standard, and streaked on Mueller-Hinton agar before the disks were applied. Ty21a grew on Mueller-Hinton agar, but had large zones. Therefore, it was tested on Mueller-Hinton agar containing 5% defribrinated sheep blood instead of Mueller-Hinton agar. All zone sizes were measured in millimeters. Chloramphenicol, ampicillin, and trimethoprim-sulfamethoxazole were included in this study because they are used in the treatment of typhoid fever (11).

Phage typing. Phage typing was done by growing the bacteria for 24 h in phage broth, diluting this broth culture to an optical density of 0.1, and using this diluted culture to flood a phage agar plate. After the plate had dried, 96 bacteriophages were applied with a multi-syringe applicator (11). Lysis patterns were recorded after 24 h.

Plating efficiency on MacConkey agar. The strains (24-h Trypticase soy agar cultures) were suspended in sterile saline (0.85% NaCl) to the density of the 0.5 McFarland standard and then serially diluted (10-fold) to 10^{-5}. We plated 0.1 ml of the 10^{-3}, 10^{-4}, and 10^{-5} dilutions on blood agar and MacConkey agar in triplicate. Colonies were counted after 1 day of incubation. The plating efficiency was determined by dividing the number of colonies on MacConkey agar by the number on blood agar.

Colonial morphology on primary isolation media. The strains were streaked to obtain isolated colonies on the following media: MacConkey, eosin methylene blue, brilliant green, Hektoen, salmonella-shigella, xylose lysine deoxycholate, tergitol 7, and bismuth sulfite agars. Colonial morphology was noted and recorded at days 1, 2, and 3.

Isolation of *S. typhi* from mixed cultures. A suspension was made in sterile physiological saline from a Trypticase soy agar slant (24 h) of one *S. typhi* strain and *E. coli* E4115 to the density of the 0.5 McFarland standard. In separate tubes, suspensions of the *E. coli* were then mixed with an equal volume of the other *S. typhi* strains. Each mixture was streaked onto the isolation media listed above (see also Table 6). The ratio of the number of *E. coli* colonies to the number of *S. typhi* colonies was determined after 1 and 2 days of incubation.

Lyophilization. The four *S. typhi* strains were freeze-dried at CDC by methods (3) previously described. The strains were grown in Todd-Hewitt broth for 18 h, harvested by centrifugation, and suspended in polyvinylpyrrolidone-inositol-sodium glutamate medium before freeze-drying. Freeze-drying was done in a VirTis mechanically refrigerated freeze-mobile unit (model 10-145 MR-BA; The VirTis Co., Inc., Gardiner, N.Y.) at −40°C, followed by 25°C. Vials were sealed under vacuum and then stored at −20°C (3). Counts were done on suspensions before and after lyophilization. In addition, a lyophilized vial of each strain was incubated for 7 days at 37°C, and a count was done.

RESULTS

Biochemical tests. The biochemical reactions of the two test strains done by conventional methods are given in Table 2. The reactions were typical for *S. typhi* except that Ty21a was H_2S negative, delayed melibiose positive, glycerol negative and galactose negative. Strain Ty21a did produce some H_2S on peptone iron agar in 7 days after it had been lyophilized by ATCC. All of the strains were identified as *S. typhi* with the kits, and the codes obtained are given in Table 3. In general, these strains can be identified biochemically with few problems.

Agglutination in salmonella antisera. The serological reactions are given in Table 4. Both strains were Vi antigen negative. There was no agglutination with O group A, B, C_1, C_2, E_1, E_2, or E_3 antiserum for either strain; therefore, these are omitted from Table 4. Ty21a grew slowly and posed problems in its serological reactions. Its agglutination (heated antigen) with O group D antiserum was weak and slightly rough before lyophilization, but there was definite agglutination compared with a control with no antiserum. After lyophilization, group D could not be detected (heated antigen), nor could the single factor O9, but there was agglutination in group D antiserum with the live antigen. Ty21a must have some galactose supplied exogenously (as occurs in vivo) to synthesize O antigens (9, 10). Since no galactose was added to the media used to grow bacteria for serology, the agglutination obtained with O antisera may not be true. After lyophilization, Ty21a had to be passed through motility medium one time before the H antigen d could be demonstrated. Serologically, H901 was the most satisfactory. It was Vi antigen negative, but the O and H antigens could be easily detected before and after lyophilization. Both strains gave better reactions with the commercial antisera when live antigens were used, which is the usual laboratory procedure. Both commercial O polyvalent and O group D antisera gave good reactions. These commercial antisera were not tested against the cultures after lyophilization. Since Ty21a and H901 were Vi antigen negative, they should be confirmed biochemically to distinguish them from other group D serotypes. *S. typhi* strains should not

produce gas from glucose and should be citrate and rhamnose negative (11).

Antibiograms. The zone sizes of the test strains are given in Table 5. The results given for Ty21a are those obtained on Mueller-Hinton with 5% sheep blood.

Phage typing. As expected, the test strains were not lysed by any of the phages, since the typing phages lyse only strains that contain the Vi antigen (11).

Plating efficiency on MacConkey agar. The plating efficiency on MacConkey agar for H901

TABLE 2. *Biochemical reaction of S. typhi strains.*

Test	Strain no.: Ty 21a	H 901
Indole	–[a]	–
Methyl red	+	+
Voges-Proskauer	–	–
Citrate (Simmons')	–	–
H_2S (TSI, LIA, PIA)	–	+
Urea	–	–
Phenylalanine	–	–
L-Lysine (Møller's)	+	+
L-Arginine (Møller's)	$+^4$	$+^4$
L-Ornithine (Møller's)	–	–
Motility	+	+
Gelatin (22°C)	–	–
KCN	–	–
Malonate	–	–
D-Glucose		
Acid	+	+
Gas	–	–
Acid from:		
Adonitol	–	–
L-Arabinose	–	–
D-Arabitol	–	–
Cellobiose	–	–
Dulcitol	–	–
Erythritol	–	–
D-Galactose	–	+
Glycerol	–	$+^2$
i-Inositol	–	–
Lactose	–	–
Maltose	+	+
D-Mannitol	+	+
D-Mannose	+	+
Melibiose	$+^4$	+
α-CH_3-glucoside	–	–
Raffinose	–	–
L-Rhamnose	–	–
Salicin	–	–
D-Sorbitol	+	+
Sucrose	–	–
Trehalose	$+^2$	+
D-Xylose	+	+
Esculin	–	–
Mucate	–	–
Tartrate (Jordan's)	$+^6$	+
DNA'ase (25° and 36°C)	–	–
$NO_3^- \rightarrow NO_2^-$	+	+
Oxidase	–	–
ONPG	–	–
Lipase (Corn oil)	–	–

[a] Symbols: –, negative at end of appropriate incubation period or 7 d; +, positive in 24 h or at time of test; the superscript gives the day the reaction became positive.

TABLE 3. *Results obtained with commercial diagnostic kits.*

Kit	Strain no.	Code obtained	Identification
API 20E	Ty 21a	4004500	*S. typhi*, melibiose$^-$
	H 901	4004540	*S. typhi*
Enterotube II	TY 21a	50040	*S. typhi*, H_2S^-
	H 901	52040	*S. typhi*
Micro-ID	TY 21a	20401	*S. typhi*, H_2S^-
	H 901	24401	*S. typhi*

TABLE 4. *Serological reactions of S. typhi strains.*

Antiserum	Agglutination of strain no.:			
	Ty 21a		H 901	
	Killed[a]	Live	Killed	Live
Commercial O polyvalent	+	+	+	+
Commercial O group D	–	+	–	+
CDC O group D	Rough	+	+	+
CDC O single factor 9	Rough	+	+	+
Commercial Vi	*b*	–		–
CDC Vi		–		–
CDC flagellar d	+		+	

[a] Antigens used with commercial O antisera were boiled for 10 min; other O antigens were heated at 60° C for 30 min. Flagellar antigens were formalinized.
[b] A blank space indicates not determined.

and the two control strains was 1, whereas for Ty21a it was 0.6.

Isolation of *S. typhi* from cultures mixed with *E. coli*. Table 6 gives the percentage of *S. typhi* present on each plating medium. *S. typhi* outnumbered *E. coli* on salmonella-shigella and Hektoen agars. These two media appear to be superior for isolating *S. typhi* when mixed with *E. coli* under these conditions. Bismuth sulfite did not appear to be a satisfactory medium for isolating *S. typhi* in 2 days; however, if held for a third day, which is often done in clinical laboratories, it may have proved more satisfactory. These strains could be mixed with *E. coli* for teaching purposes. However, Ty21a took 2 days to grow on MacConkey and tergitol 7 when mixed with *E. coli*, and it did not grow on

TABLE 5. *Antimicrobial susceptibilities of S. typhi strains.*

Antibiotic	Zone size (mm) of strain no.:	
	Ty 21a	H 901
Ampicillin	28	25
Carbenicillin	28	27
Cephalothin	21	25
Chloramphenicol	30	25
Colistin	15	13
Gentamicin	28	23
Kanamycin	25	23
Nalidixic acid	18	20
Penicillin	12	16
Streptomycin	15	16
Tetracycline	21	23
Trimethoprim + Sulfamethoxazole	34	32

TABLE 6. *Isolation of S. typhi strains mixed with E. coli.*

Plating medium	Percent of mixture at 24 h that was *S. typhi* for:	
	Ty 21a	H 901
Blood agar	5	20
Brilliant green	0	20
Eosin methylene blue	10	10
Hektoen	90	100[b]
MacConkey	0[a]	
Salmonella-Shigella	90	90
Tergitol 7	0[a]	100
Xylose-lysine deoxycholate	0	0
Bismuth sulfite[c]		

[a] Five percent at 48 h.
[b] Neither *E. coli* nor *S. typhi* grew in 48 h.
[c] Colonies were too small to count in 48 h.

TABLE 7. *Summary of characteristics of S. typhi strains.*

Characteristic	Strain no.:	
	Ty 21a	H 901
Biochemical tests	Satisfactory (H_2S^-)	Satisfactory
Commercial identification kits	Satisfactory	Satisfactory
Serology:		
O Killed	Rough	Satisfactory
Live	Satisfactory?	Satisfactory
Vi	Negative	Negative
H	Weaker after lyophilization	Satisfactory
Plating efficiency (MacConkey)	0.6	1.0
Preferred differential plating media	SS and Hektoen	SS and Hektoen
Colonial morphology on plating media	Typical but small	Typical
Post-lyophilization counts	Satisfactory	Satisfactory
Phage type	W form	W form

[a] SS = Salmonella-Shigella agar.

brilliant green in 2 days. Neither *E. coli* nor H901 grew on MacConkey agar in this experiment. We did not consider it necessary to repeat the MacConkey agar, since the mixtures containing the control strains of *S. typhi* gave 90% *E. coli* and 10% *S. typhi* on MacConkey agar, and H901 grew in the same proportions as the control strains on the other media tested.

Lyophilization. The counts before and after lyophilization and after exposure of vials to 37°C for 1 week were satisfactory for the control and test strains; none of the counts after lyophilization were below 1×10^8 colony-forming units per ml.

DISCUSSION

A summary of the characteristics of both strains is given in Table 7. The strains evaluated in this study can be readily identified biochemically even though Ty21a was H_2S negative. Serologically, however, Ty21a could pose problems for less experienced microbiologists and students. Ty21a grew poorly, and its plating efficiency was lower. These strains were susceptible to the antimicrobial agents commonly used to treat typhoid fever. Strains of *S. typhi* used in teaching must be susceptible to these antibiotics.

No virulence tests of any strains were done. The numbers of bacteria and conditions used in reported virulence studies may not be comparable to conditions in the laboratory. In addition, these strains could revert to more virulent forms after many passages or other manipulations. If these strains are used for educational purposes, they must be treated as if they are fully virulent strains. There is no substitute for good laboratory safety practices.

It has been our experience that some of the lyophilized vials in which proficiency or "unknown" bacteria have been sent are difficult to handle, particularly vials that require introduction of a liquid into the vial with a syringe and needle. We have often had difficulty with the suspension of bacteria dripping when the needle was withdrawn. Vials designed with rubber stoppers and metal rims that can be completely removed would eliminate the use of syringes, since pipettes could be used. There are devices available that can be used to remove metal rims from vials. One such device, called a decapper, can be purchased from Wheaton Scientific, Millville, N.J., and The West Company, Phoenixville, Pa. Vials that have to be broken open are also hazardous because of the release of freeze-dried cells. All freeze-dried vials must be opened in a safety cabinet with a supply of disinfectant on hand.

These strains have been freeze-dried by ATCC in skim milk in double-vial preparations. These vials should be opened according to the instructions that accompany them. We recommend that these strains be reconstituted in Trypticase soy broth or a comparable broth medium and streaked onto blood agar to obtain maximum growth. We recommend that the whole culture and a single colony be frozen in 10% skim milk (−40 to −70°C) for long-term preservation and also be maintained on Pai medium at room temperature (11).

H901 appears to be the most typical for teaching purposes except that it is Vi antigen negative. The selection of either of these *S. typhi*

strains would be determined by its particular teaching purpose. Therefore, we do not recommend either strain as the best available one, but merely give the advantages and disadvantages of each. O901 was previously deposited in ATCC (ATCC 10749). This strain should not be confused with those that we have evaluated and deposited. A third strain, 42-A-58V, that was isolated from a carrier in Panama has been reported to have reduced virulence for mice (1, 8). Previous virulent strains from this carrier had been used for a vaccine (4, 8). Its reduced virulence was attributed to its growth requirement for xanthine, which is a purine. We were unable to obtain this strain for this study, but we plan to evaluate it in the future. It is Vi antigen positive. No reports indicate that it has been tested in humans. Since this study began, R. Brown, Sierra Valley District Hospital, Loyalton, Calif., and B. Stocker, Department of Medical Microbiology, Stanford University School of Medicine, Stanford, Calif., have informed us that they have genetically constructed a group of *S. typhi* strains that are expected to have reduced virulence. These strains need a complete laboratory evaluation. Perhaps these strains will prove more satisfactory for teaching purposes. For now, the two strains evaluated here together with good laboratory safety practices offer the best compromise for using *S. typhi* in teaching and proficiency tests. We hope that a more definitive recommendation can be made in the future.

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ADDENDUM IN PROOF

After this study was completed, B. Stocker informed us that he and R. Brown had tested our stock cultures of strains Ty21a and H901 for virulence in mice and had compared them to the virulent, Vi antigen-positive strain Ty2. Ty2 was at least 10 times more virulent (based on 50%-lethal-dose data) than Ty21a and H901.

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