# Differentiation of *Vibrio cholerae* O1 Isolates with Biochemical Fingerprinting and Comparison with Ribotyping

**M Ansaruzzaman[1], M John Albert[1] I Kühn[2], SM Faruque[1], AK Siddique[1], and R Möllby[2]**

*[1]International Centre for Diarrhoeal Disease Research, Bangladesh, GPO Box 128, Dhaka 1000, Bangladesh; [2]Microbiology and Tumor Biology Center, Karolinska Institute, S-171 77, Stockholm, Sweden*

## ABSTRACT

The Phene Plate (PhP) system is a commercially available typing system based on the measurements of kinetics of selected biochemical reactions of bacteria grown in liquid medium in 96-well microplates. The system uses numerical analysis to identify biochemical phenotypes among the tested strains. In the present study, a set of 16 discriminatory tests were used to differentiate 117 strains of *Vibrio cholerae* O1 from Mexico and Bangladesh. The stability of PhP types of 16 isolates under different storage temperatures and after repeated subcultures were also evaluated. The PhP system had a reproducibility of 95%. Storage either at +4 ° C or -70 ° C, did not affect the reactions of the isolates, whereas 4 strains (25%) stored at room temperature and 5 strains (31%) subjected to 30 consecutive subcultures, exhibited minor changes in their biochemical reactions. Endemic isolates of *V. cholerae* O1 from Bangladesh were more diverse (diversity index = 0.84 to 0.93) than epidemic isolates from Mexico (diversity index = 0.73). Using a collection of 33 heterogeneous isolates of classical biotype of vibrios, PhP typing and ribotyping were compared. PhP typing discriminated more types (n=23) than ribotyping (n=5), whereas a combination of both yielded 27 types. The PhP system appears to be a simple, reliable and highly discriminating method for typing of *V. cholerae*, and may prove especially useful as a first screening method in epidemiological studies of *V. cholerae*.

***Key words*:** *Vibrio cholerae*; Phenotypes

## INTRODUCTION

*Vibrio cholerae* belonging to serogroups O1 and O139 are the causative agents of cholera (1). There are two biotypes of *V. cholerae* O1, classical and El Tor, and each biotype can be further subdivided into two serotypes, Inaba and Ogawa (2). Suitable typing methods with a wide appeal for further discrimination of biotypes and serotypes for epidemiological studies have recently become available. These subtyping methods include ribotyping (3), ctxA genotyping (4), pulsed field gel electrophoresis (PFGE) (5), and multilocus enzyme electrophoresis (MEE) (6). The first three are DNA typing methods, among which, in ribotyping, strains are differentiated based on restriction fragment length polymorphism (RFLP) of ribosomal RNA genes using rRNA gene probes (7). MEE is based on differences in the migration of a set of enzymes that correspond to allelic differences among isolates (6). Although these subtyping methods may be sufficiently discriminatory, they are laborious and expensive, and hence may not be suitable for a large collection of isolates.

The PhenePlate or PhP system is an automated system for biochemical fingerprinting of bacterial isolates, which is based on numerical analysis of the speed of colour changes of several biochemical reactions (8). It is a commercially available system that is simple to use, discriminatory and reproducible, and is suitable for large investigations involving hundreds of isolates. The system was previously used for epidemiological and ecological studies of different groups of bacteria (9-11,21).

In the present study, we have evaluated the PhP system for typing *V. cholerae* O1 isolates. The performance of the PhP system was compared with ribotyping.

## MATERIALS AND METHODS

### *V. cholerae* O1 isolates

The isolates studied were from epidemic and endemic cases of cholera in humans. A total of 117 isolates were studied from the following five collections ( Table I):

**Table 1.** *V. cholerae* collections studied

| Collection no. | No. of isolates | Origin of isolates | Biotype | Serotype | Diversity Index |
|---|---|---|---|---|---|
| I | 24 | Mexico 1991 | El Tor | Inaba | 0.73 |
| II | 22 | Bangladesh 1992 | El Tor | Ogawa | 0.84 |
| III | 22 | Bangladesh 1974-1992 | El Tor | Ogawa/Inaba | 0.93 |
| IV | 33 | Bangladesh 1961-1992 | Classical | Ogawa/Inaba | 0.97 |
| V | 16 | Bangladesh 1993 | El Tor | Ogawa | 0.65 |

*Collection I*: Twenty-four isolates of *V. cholerae* O1 biotype El Tor, serotype Inaba, were obtained from the outbreak of cholera in Mexico in 1991.

*Collection II*: Twenty-two isolates of *V. cholerae* O1 biotype El Tor, serotype Ogawa, were obtained from field cases of endemic cholera in southern Bangladesh in 1992.

*Collection III*: Twenty-two isolates of *V. cholerae* O1 biotype El Tor, both Ogawa and Inaba serotypes, from endemic cholera cases, who attended the hospital of the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B), located in Dhaka during 1974- 1992.

*Collection IV*: Thirty-three isolates of *V. cholerae* O1 belonging to classical biotype and both serotypes isolated from cholera patients who attended the ICDDR,B hospital in Dhaka, and from field cholera cases in southern Bangladesh, between 1961 and 1992. All of these isolates have been included in a ribotyping study previously (7).

Collections I and II were stored at -70° C in Trypticase soy broth (Gibco, NY, USA) with 15% glycerol, and collections III and IV were stored as lyophilised cultures until studied.

*Collection V:* Sixteen fresh isolates of *V. cholerae* O1 biotype El Tor, serotype Ogawa, from primary culture plates of taurocholate-tellurite-gelatin agar (TTGA) (12) inoculated with stool from endemic cholera cases, who attended the ICDDR,B hospital in Dhaka during January 1993. These isolates were included for stability test.

All isolates were reconfirmed as *V. cholerae* O1 by standard procedures (13). The procedures included biochemical reactions in motility-indole-urease medium and Kligler's iron agar, slide agglutination with polyvalent and serotype-specific antisera (ICDDR,B), and biotyping by chicken cell agglutination, polymyxin B susceptibility, and susceptibility to Mukherjee's classical and El Tor phages.

### Biochemical fingerprinting with the PhP system

The PhP-VC plate (commercial supplier is BioSys inova, S-11351 Stockholm, Sweden) consists of 96-well microtitre plates with six sets of 15 substrates each. These 15 substrates were carefully selected from a larger set of 96 substrates for being those showing the highest discrimination and reproducibility among 80 unrelated *V. cholerae* isolates (all El Tor isolates; these were obtained from Africa in 1991, Peru in 1992 and Bangladesh during 1961-1992. The identity level (see below) obtained with these isolates was used for evaluating other strain collections used in the present study).

The 15 substrates used were: 1: D-galactose; 2: maltose; 3: D-trehalose; 4: D-lactose; 5: inositol; 6: glycerol; 7: L-rhamnose; 8: ß-methyl-D-glucoside; 9: D-gluconate; 10: D-mannitol; 11: potato starch; 12: glycogen; 13: fumarate; 14: pyruvate; 15: succinate; and the 16th well contained medium control with pH 8.2.

The stock culture was subcultured on a nutrient agar plate, and incubated at 37 ° C for 18-24 hours. One colony measuring approximately 2 mm was inoculated into 10 ml medium containing 0.1% (w/v) Bactopeptone (Difco, Detroit, MI, USA), 1.0% (w/v) sodium chloride, and 0.01% (w/v) bromothymol blue (pH 8.5). The bacterial suspension was added to a pre-prepared PhP-VC plate containing dehydrated substrates with the aid of a multi-channel pipette delivering 0.15 ml of broth culture into each well (a 96-well microtitre plate containing six sets of reagents could be used to test six different isolates.) To allow for proper rehydration of substrates, plates were stored at 4 ° C overnight, and then incubated at 37 ° C the following morning. On use of the substrate in a well, the colour of the bromothymol indicator changed. The absorbance of each reaction was measured at a wavelength of 620 nm in a microplate reader (Dynatech MR5000, Chantilly, VA, USA), at 7, 24, and 48 hours. The absorbance values were electronically transferred to a personal computer, multiplied by a factor of 10, and stored as integer values. After the last measurement at 48 hours, the mean value of all three readings was calculated for each reaction (Table IIa for an example). The biochemical fingerprint of an isolate thus consists of 16 quantitative numbers, each one ranging from 0 (acidic reaction = yellow colour on all measurements) to 30 (alkaline reaction = blue colour on all measurements) (Table IIb for an example).

**Table IIa.** Test results (biochemical fingerprins) from a *V. cholerae* isolate measured at three incubation times

| Incubation time (h) | Substrate number | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 |
| 7 | 12 | 6 | 4 | 21 | 4 | 17 | 21 | 7 | 4 | 4 | 8 | 5 | 18 | 22 | 8 | 20 |
| 24 | 4 | 5 | 3 | 21 | 4 | 4 | 21 | 3 | 4 | 4 | 8 | 9 | 18 | 26 | 9 | 24 |
| 48 | 3 | 5 | 4 | 21 | 4 | 3 | 21 | 3 | 3 | 3 | 9 | 7 | 18 | 26 | 24 | 26 |
| Mean | 6 | 5 | 4 | 21 | 4 | 8 | 21 | 4 | 4 | 4 | 8 | 7 | 18 | 25 | 14 | 23 |

**Table IIb.** Biochemical fingerprints of five *V. cholerae* isolates

| Isolate no. | Biochemical fingerprint: Substrate number | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 |
| 1* | 6 | 5 | 4 | 21 | 4 | 8 | 21 | 4 | 4 | 4 | 8 | 7 | 18 | 25 | 14 | 23 |
| 2 | 7 | 5 | 13 | 15 | 5 | 11 | 22 | 9 | 4 | 5 | 14 | 8 | 22 | 23 | 12 | 24 |
| 3 | 9 | 6 | 11 | 14 | 6 | 10 | 22 | 9 | 5 | 8 | 8 | 7 | 20 | 22 | 6 | 20 |
| 4 | 14 | 6 | 6 | 23 | 12 | 18 | 24 | 8 | 15 | 10 | 20 | 14 | 23 | 29 | 26 | 28 |
| 5 | 12 | 5 | 5 | 21 | 11 | 16 | 21 | 8 | 14 | 11 | 20 | 13 | 21 | 27 | 24 | 27 |

*The data for isolate 1 are extracted from Table IIa

**Table IIc.** Similarity matrix obtained from pair-wise comparisons of the biochemical fingerprints in Table IIb. The similarities were calculated as correlation-coefficients

| Isolate no. | Correlation-coefficient compared to isolate no. 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| 1 | - | | | | |
| 2 | 0.90 | - | | | |
| 3 | 0.87 | 0.93 | - | | |
| 4 | 0.89 | 0.78 | 0.67 | - | |
| 5 | 0.87 | 0.77 | 0.64 | 0.99 | - |

PhP types found: Isolates 1, 2 and 3 are single (S) types; isolates 4 and 5 belong to the same common (C) type if identity level is at correlation-coefficient >0.965

**Table III.** Comparison of stabilities of PhP types of 16 *V. cholerae* O1 isolates when stored for 30 days at different temperatures and after repeated subculture

| Comparison between Sets | Mean similarity | No. of isolates identical |
|---|---|---|
| 1 and 2 | 0.980 | 16 |
| 1 and 3 | 0.980 | 15 |
| 1 and 4 | 0.976 | 15 |
| 2 and 3 | 0.974 | 12 |
| 2 and 4 | 0.972 | 11 |
| 3 and 4 | 0.975 | 13 |

Set1: Isolates stored at +4 °C
Set2: Isolates stored at -70 °C
Set3: Isolates stored at room temperature, 25 °C
Set4: Isolates subcultured 30 times

The biochemical fingerprints of N isolates within a collection were compared pair-wise, and the similarity between each pair was calculated as the correlation- coefficient. This yielded a similarity matrix consisting of N x (N-1)/2 correlation coefficients (Table IIc for an example). The similarity matrix (Table IIc) was clustered according to the unweighted-pair group method with arithmetic averages (UPGMA) to yield a dendrogram (for an example, see Fig. 1) (14). The reproducibility of the PhP-VC plate was evaluated by assaying 24 of the above isolates in duplicate in the same assay. The level of identity (the ID level) between isolates was defined as the mean of the correlation-coefficients obtained between these duplicate assays minus two standard deviations (SD) of this mean (11) . PhP types consisting of more than one isolate were named C (common) types, whereas those consisting of only one isolate were named S (single) types.

All data handling, including optical readings, calculations of correlation-coefficients, diversity indices, as well as clustering and printing of dendrograms were performed using the PhP software (BioSys inova, Stockholm, Sweden), which is commercially available.

**Stability of fingerprints upon storage at different temperatures and after subculture**

The 16 fresh isolates of *V. cholerae* (collection V) were used for these tests. After confirmation of the colonies as *V. cholerae* O1 by biochemical reactions and slide agglutination test with specific antiserum, single isolated colony from each strain was subcultured onto nutrient agar (NA) plates and incubated at 37 ° C. From this first subcultured NA plate, three different sets of stock cultures were prepared. Set 1 was stored at 4 ° C on NA plates; set 2 was stored at -70 ° C in Luria broth containing 15% (v/v) glycerol; set 3 was stored at room temperature (25 ° C) in T1N1 soft agar. Finally, 30 consecutive subcultures were made from the first subcultured NA plate by transferring a single colony to a new agar plate every day. The 30th subculture was marked as set 4. Finally, from all four sets, subcultures were made once on nutrient agar, and after 20 hours incubation, the growth was used for biochemical fingerprinting, which was done in duplicate and blindly.

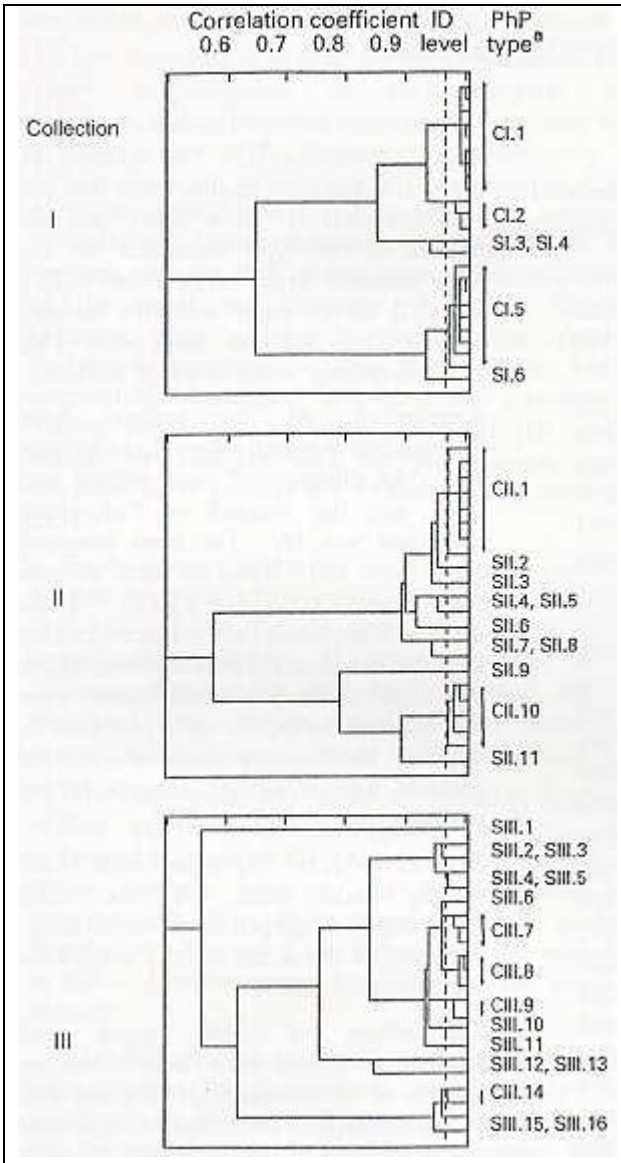


**Fig. 1.** Three dendrograms showing the relationship among the PhP type of V cholerae 01 isolates from three different collections. Dotted lines indicate the level of identity (correlation-coefficient >0.965).

**Collection I:** Twenty-four E1 Tor V.cholerae 01 isolates obtained from the epidemic cholera in 1991 in Mexico.

**Collection I:** Twenty-four E1 Tor V.cholerae 01 isolates obtained from the epidemic cholera cases in southern Bangladesh in 1992.

**Collection I:** Twenty-four E1 Tor V.cholerae 01 isolates obtained from the epidemic cholera cases seen at the Dhaka hospital,Bangladesh,during 1974-1992.

**Ribotyping**

The rRNA gene probe used for ribotyping was a *Bam*HI fragment of a cloned *Escherichia coli* rRNA operon obtained from the recombinant plasmid pKK3535 (15). The recombinant plasmid was purified and digested with *Bam*HI, and the insert was purified by electroelution from agarose gel as described by Maniatis *et al*. (16). The insert (probe DNA) was labelled by random priming (17) with [a -$^{32}$P]deoxycytidine triphosphate (3000 Ci/mmol, Amersham International plc., Aylesbury, United Kingdom) and a random primer DNA labelling system (Bethesda Research Laboratories, Gaithersburg, MD, USA). Southern blots of purified bacterial chromosomal DNA after digestion with restriction enzymes *Bg*lI and *Hind*III (Bethesda Research Laboratories) were hybridised with labelled rRNA probe, washed under stringent conditions, and autoradiographed as described previously (18). The ribotyping data on 33 classical biotype of *V. cholerae* O1 (listed as collection IV in the section Materials and Methods) were extracted from a previous publication (7).

**Comparison between PhP and ribotyping**

The performances of PhP typing and ribotyping were compared using the 33 isolates in collection IV.

The isolates were typed with both methods, and assigned into types. The discriminatory power of each typing method was calculated as Simpson's diversity index (Di) according to the formula: Di = 1 -S [ni x (ni - 1)/(N x (N - 1)] (19), where ni is the number of isolates in the ith type, and N is the total number of isolates studied (in this case 33). The value of this index depends both on the number of different types identified and on how even the distribution of isolates into different types is. It is high (maximum value 1.0) if most isolates belong to different types, and is low (minimum value 0) if one type is dominating.

## RESULTS

### Reproducibility of the PhP system

The intra-assay reproducibility was estimated by duplicate assays of the 33 isolates obtained from collection IV. The mean similarity among the isolates was 0.987, the standard deviation was 0.011, and thus, the identity level was set at a correlation-coefficient of 0.965. This yields a reproducibility of >95% (16), i.e. of 100 comparisons between identical strains, more than 95 appear as identical.

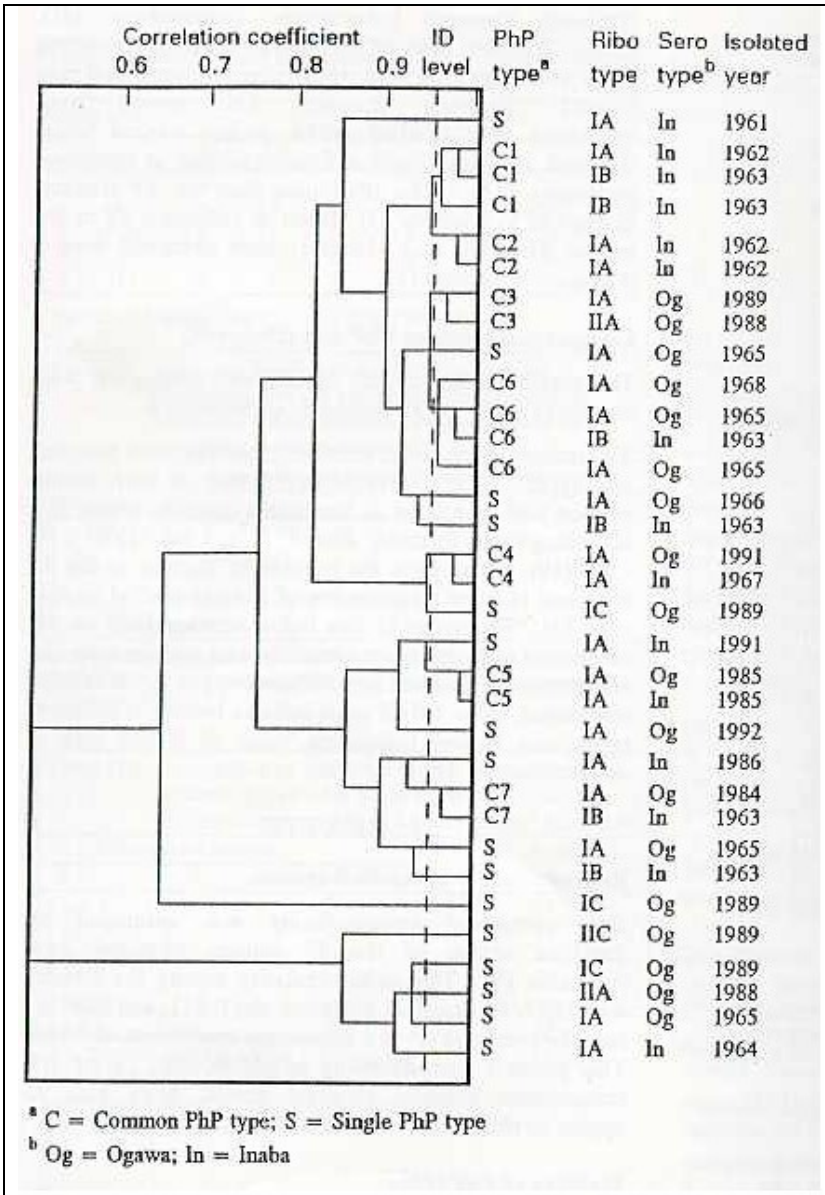


**Fig. 2.** Dendrogram showing the relationship among the PhP type of 33 classical biotype V. cholerae 01 isolates from a heterogeneous collection obtained during 1961-1992 (collection IV) in Bangladesh. Dotted line indicates the level of identity. The figures also show the ribotype and serotype of each isolate.

### Stability of PhP types

The stability of PhP types of 16 freshly isolated *V. cholerae* O1 strains (collection V) under different temperatures of storage, and after repeated subculturing, was studied. The results are shown in Table III. When isolates of the same strain stored at 4 $^{o}$C and -70 $^{o}$C were compared, all yielded identical biochemical fingerprints and correlation-coefficients above the identity level. The lowest similarity was obtained when subcultured isolates were compared to those stored at -70 $^{o}$C (only 11 of 16 identical). In total, 82 of 96 (84%) comparisons between the same isolates yielded the same PhP types, and in all the other 14 cases, the PhP types were still similar, showing a similarity of >0.95.

### Typing of *V. cholerae* O1 by PhP system

*Mexican isolates* (Fig. 1, collection I): As expected of an epidemic, these isolates showed a low diversity (Di = 0.73), and only six different PhP types were found. Eighteen of the isolates consisted of two dominant PhP types (CI.1 and CI.5), three isolates belonged to a PhP type similar to CI.1 (type CI.2) and another isolate was similar to PhP type CI.5 (SI.6).

*Bangladeshi El Tor isolates from field (*Fig. 1*,* collection II): The diversity of these isolates was higher than for the Mexican isolates (Di = 0.84), and the number of PhP types identified was 11. Two common types were found (CII.1 and CII.10) in eight and five isolates respectively, and the other nine PhP types were only found in single isolates.

*Bangladeshi El Tor isolates from hospitalised patients* (Fig. 1, collection III): The diversity of these isolates was 0.93, and the number of PhP types identified was 16. The most common types were only found in three isolates (PhP types CIII.7 and CIII.8). Thus, these isolates were more heterogeneous than the Bangladeshi field isolates, which was expected since the field isolates were all collected during the same year (1992), whereas the isolates from hospitalised patients were collected over a longer period of time (1974 to 1992).

The above three sets were tested twice blindly under code, and the same biochemical fingerprints and correlation- coefficients above the identity level were obtained both times.

*Comparison of PhP typing and ribotyping*: Thirty-three classical biotype strains of *V. cholerae* O1 (Ogawa and Inaba serotypes) were studied, and their ribotype data were extracted from a previous publication (4). A comparison of PhP types, ribotypes and serotypes is shown in Fig. 2. The isolates fell into five different ribotypes, and 23 different PhP types. A combination of PhP typing and ribotyping yielded 27 different types. If serotyping was also included, 29 different types were found, and only three types containing more than one isolates were found (two isolates of PhP type C1, both isolates of PhP type C2, and three isolates of PhP type C6). In two of these types the isolates were also from the same year, which indicated that they might be of the same clone. The discriminatory power as defined by Di was 0.51 for serotyping, 0.58 for ribotyping, 0.97 for PhP typing and 1.0 for the combination of all three methods.

## DISCUSSION

Earlier, biotyping was used as one of the techniques for strain differentiation in epidemiological studies. However, this technique had limitations, because too few substrates were used. Moreover, reactions were scored only qualitatively as either positive or negative at the end of the incubation period, and there was no accurate way of assessing intermediate results. Biochemical fingerprinting with the PhP system uses quantitative measures of the speed and intensity of several biochemical reactions, thus making it more discriminatory than traditional biotyping. The PhP system has been successfully developed and used for subtyping of a variety of bacteria for epidemiological and ecological studies (9-11,20,21), and the performance has been shown to be as good as that of DNA typing methods (20, 22, 23).

In the present study, we developed and evaluated the performance of the PhP system for typing *V. cholerae* O1. Our data indicate that the system is discriminatory and reproducible. Stability of fingerprints after storage at different temperatures and after repeated subcultures suggested that optimal results were achieved by preserving the isolates either at +4 $^{o}$C or -70 $^{o}$C.

The PhP system was further evaluated by applying the system to type epidemiologically well-characterised isolates of *V. cholerae* O1. As expected, isolates from the recent cholera epidemic in Mexico were more homogeneous than those from endemic cholera in Bangladesh. Moreover, the PhP system was reproducible as identical results were obtained when the assays were repeated.

The PhP system was compared with an established typing system for *V. cholerae*, i.e. ribotyping. It was found that the discrimination achieved by the PhP system was higher than that obtained by ribotyping. However, some degree of correlation between the two typing systems was observed since, when the isolates belonged to the same PhP type, they usually also belonged to the same ribotype. The main advantages of PhP typing, as compared to DNA typing are its simple performance and automatic data evaluation and presentation. It is, thus, a very convenient system for studies of large numbers of bacteria.

Other advantages of the PhP system are that numerical data of high precision are generated enabling cluster analysis, and new and old data out of the computer memory can also be easily compared. In contrast, molecular typing methods based on electrophoresis may result in reproducibility problems and difficulties in comparing data generated on different gels. These methods are, thus, mostly restricted to investigations where only a few strains need to be compared. For more careful epidemiological investigations of many isolates, a combination of methods should be useful, where PhP typing is used as a first screening method and suitable DNA typing methods are applied to a selected number of isolates based on their biochemical fingerprints.

## ACKNOWLEDGEMENTS

This research was supported by the Swedish Agency for Research and Co-operation (SAREC) and the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B). The ICDDR,B is supported by countries and agencies which share its concern for the health problems of developing countries. Current donors include: the aid agencies of the Governments of Australia, Bangladesh, Belgium, Canada, China, Denmark, Germany, Japan, the Netherlands, Norway, Republic of Korea, Saudi Arabia, Sri Lanka, Sweden, Switzerland, Thailand, the United Kingdom and the United States; international organisations including the Arab Gulf Fund, the Asian Development Bank, European Union, the United Nations Children's Fund (UNICEF), the United Nations Development Programme (UNDP), the United Nations Population Fund (UNFPA) and the World Health Organisation (WHO); private foundations including Aga Khan Foundation, Child Health Foundation, Ford Foundation, Population Council, Rockefeller Foundation and the Sasakawa Foundation; and private organisations including American Express Bank, Bayer AG, CARE, Family Health International, Helen Keller International, the Johns Hopkins University, Macro International, New England Medical Centre, Procter Gamble, RAND Corporation, SANDOZ, Swiss Red Cross, the University of Alabama at Birmingham, the University of Iowa, and others.

We thank Dr. J.S. Castillo for provision of the Mexican vibrio isolates, and Mr. Manzurul Haque and Mr. Md. Ali Arshad Meah for secretarial assistance.

## REFERENCES

1. Albert MJ, Ansaruzzaman M, Bardhan PK, et al. Large epidemic of cholera-like disease in Bangladesh caused by *V. cholerae* O139 synonym Bengal. *Lancet* 1993;342:389-90.
2. Janda JM, Powers C, Bryant RG, Abbott SL. Current perspectives on the epidemiology and pathogenesis of clinically significant *Vibrio* spp. *Clin Microbiol Rev* 1988;1:245-67.
3. Popovic T, Bopp CA, Olsvik O, Wachsmuth K. Epidemiologic application of a standardized ribotype scheme for *Vibrio cholerae* O1. *J Clin Microbiol* 1993;31:2474-82.
4. Wachsmuth IK, Bopp CA, Fields PI. Difference between toxigenic *Vibrio cholerae* O1 from South America and the U.S. Gulf coast. *Lancet* 1991;1:1097-8.
5. Cameron DN, Khambaty FM, Wachsmuth IK, Tauxe RV, Barrett TJ. Molecular characterization of *Vibrio cholerae* O1 strains by pulsed-field gel electrophoresis. *J Clin Microbiol* 1994;32:1685-90.
6. Salles CA, Momen H. Identification of *Vibrio cholerae* by enzyme electrophoresis. *Trans R Soc Trop Med Hyg* 1991;85: 544-7.
7. Faruque SM, Alim ARMA, Rahman MM, Siddique AK, Sack RB, Albert MJ. Clonal relationships among classical *Vibrio cholerae* O1 strains isolated between 1961 and 1992 in Bangladesh. *J Clin Microbiol* 1993;31:2513-6.

8. Möllby R, Kühn I, Katouli M. Computerised biochemical fingerprinting - a new tool for typing of bacteria. *Rev Med Microbiol* 1993;4:231-41.

9. Katouli M, Kühn I, Lund A, Wallgren P, Söderlind O, Möllby R. Phenotypic characterization of the intestinal *Escherichia coli* of pigs during suckling, post-weaning and fattening periods. *Appl Environ Microbiol* 1995;61:778-83.

10. Kühn I, Allestam G, Stenström TA, Möllby R. Biochemical fingerprinting of water coliform bacteria, a new method for measuring the phenotypic diversity and for comparing different bacterial populations. *Appl Environ Microbiol* 1991;57:3137-77.

11. Kühn I, Brauner A, Möllby R. Evaluation of numerical typing systems for *Escherichia coli* using the API50CH and the PhP-EC systems as models. *Epidemiol Infect* 1990;105:521-31.

12. Monsur KA. A highly selective gelatin-taurocholate-tellurite medium for the isolation of *Vibrio cholerae*. *Trans R Soc Trop Med Hyg* 1961;55:440-2.

13. Kay BA, Bopp CA, Wells JG. Isolation and identification of *Vibrio cholerae* O1 from fecal specimens. *In:* Wachsmuth IK, Blake PA, Olsvik O, editors. *V. cholerae* and cholera: molecular to global perspectives. Washington DC: American Society for Microbiology, 1994: 3-25.

14. Sneath PHA, Sokal RR. Numerical taxonomy. New York: Freeman, 1973.

15. Brosius J, Ullrich A, Raker MA, *et al.* Construction and fine mapping of recombinant plasmids containing the rrnB ribosomal RNA operon of *Escherichia coli*. *Plasmid* 1981;6:112-8.

16. Maniatis T, Fritsch EF, Sambrook J. Molecular cloning: a laboratory manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory, 1982.

17. Feinberg A, Vogelstein B. A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. *Anal Biochem* 1984;137:266-7.

18. Stull TL, LiPuma JJ, Edlind TD. A broad spectrum probe for molecular epidemiology of bacteria: ribosomal RNA. *J Infect Dis* 1988;157:280-6.

19. Hunter PR, Gaston MA. Numeric index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. *J Clin Microbiol* 1988;26:2465-6.

20. Kühn I, Huys G, Jansen P and Kersters, K. Diiversity and stability of coliforms and *Aeromonas* in water from a well, studied over four years of time. *Canadian J Microbiol* 1997 (in press).

21. Kühn I, M. Albert J, Ansaruzzaman M, Bhuiyan NA, Alabi SA, Huys G, *et al.* Hybridization groups, genotypes and virulence factors among Aeromonas strains isolated from patients with diarrhoea, from healthy controls and from environmental sources in Bangladesh. *J Clin Microbiol* 1997 (in press).

22. Kühn I, Burman L.G, Haeggman S, Tullus K, Murray B.E. Biochemical fingerprinting for epidemiological typing of enterococci compared with ribotyping and pulsed field gel electrophoresis of DNA. *J Clin Microbiol* 1995;33:2812-2817.

23. Kühn I, Austin DA, Austin B, Blanch AR, Grimont PAD, Jofre J, *et al.* Diversity of *Vibrio anguillarum* isolates from different geographical and biological habitats, determined by the use of a combination of eight different typing methods. *System Appl Microbiol* 1996;19:442-450.