TRANSITION OF DRUG SUSCEPTIBILITIES OF *VIBRIO CHOLERAE* O1 IN LAO PEOPLE'S DEMOCRATIC REPUBLIC

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Abstract. The changes of drug susceptibilities of *Vibrio cholerae* O1 isolated during the past 7 years (1993-1999) in Lao PDR were investigated. The most noteworthy finding was the appearance of polymyxin B sensitive El Tor vibrios. Until 1996, the susceptibilities were almost as expected and cholera disappeared in 1997. When a cholera outbreak resurfaced in 1998, the susceptibilities of isolated *V. cholerae* O1 against tetracycline, sulfamethoxazol-trimethoprim, chloramphenicol and polymyxin B were quite different from those of previously isolated organisms. Minimum inhibitory concentrations (MICs) of tetracycline and chloramphenicol against the isolates in 1998 were about 16 times higher than those against the previous isolates, and the MICs of sulfamethoxazol-trimethoprim were about 256 times higher than those against the previous isolates, (trimethoprim 32 μg/ml: sulfamethoxazol 608 μg/ml). Eleven percent of the isolates (11/99) were as sensitive to polymyxin B as the classic cholera vibrios (MIC < 2 μg/ml). In 1999, the susceptibility pattern was almost the same as that in 1998 except for polymyxin B to which 58% of the isolates (21/36) became sensitive.

INTRODUCTION

Vibrio cholerae Ol, the causative agent of cholera, are usually sensitive to therapeutic antimicrobials (Higa *et al*, 1995). Although the emergence of tetracycline resistant *V. cholerae* O1 has been occasionally reported (Kobari *et al*, 1970; Mhalu *et al*, 1979; Glass *et al*, 1980; Ehara *et al*, 1984; Threlfall *et al*, 1993; Yamamoto *et al*. 1995), it has not become a serious problem, since some other drugs can be substituted for tetracycline. The resistant strains usually disappeared spontaneously or after preventive medication was stopped. However, an epidemic due to the resistant strains in Tanzania during 1977 was thought to last for a substantially long period (Ehara *et al*, 1984). The duration of cholera diarrhea is 5 to 7 days when the patients are treated with fluid infusion, but additional antimicrobials have been shown to shorten the duration to fewer days (Hischhorn *et al*, 1974). Since shortening of the symptomatic period is a positive merit of the antimicrobial treatment, the drug sensitivity pattern of these pathogens has been monitored to provide a proper treatment for the patients. In Lao People's Democratic Republic (Lao PDR), a cholera epidemic started in 1993 after a long interval free from the disease. Although the pathogens were sensitive to the therapeutic antimicrobials as expected until 1996, a change of drug sensitivity pattern started in 1998 (Iwanaga *et al*, 2000). In this communication, we have reported the transition of drug susceptibilities of *V. cholerae* O1 isolated in Lao PDR during the past 7 years.

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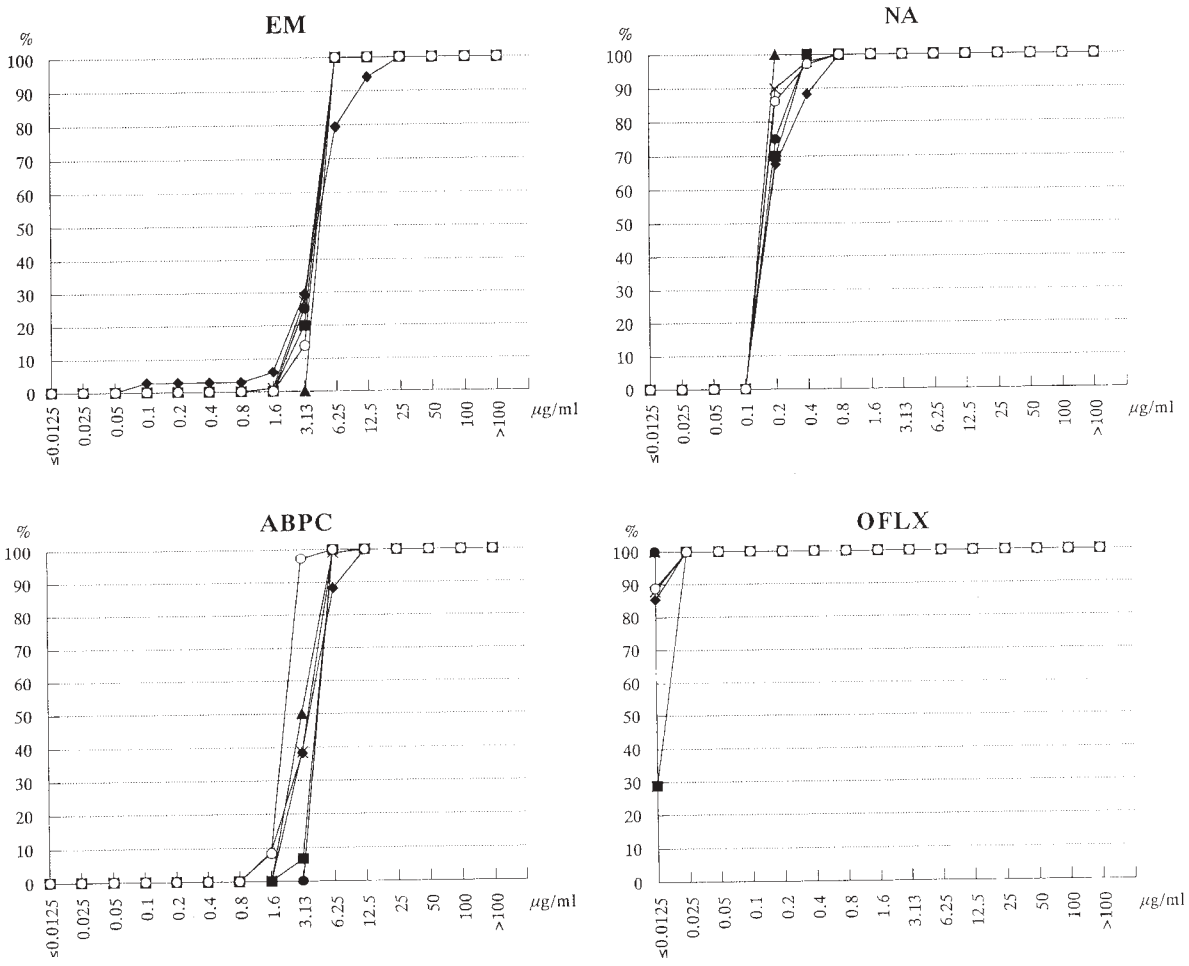
MATERIALS AND METHODS

Bacterial strains

V. cholerae O1 strains isolated from cholera patients in a variety of Lao PDR districts since 1993 were examined. The isolates were identified as *V. cholerae* O1 by routine laboratory



Figs 1 - 8–MIC distributions as expressed by cumulative percent.
EM: erythromycin, ABPC: ampicillin, NA: nalidixic acid, OFLX: ofloxacin,

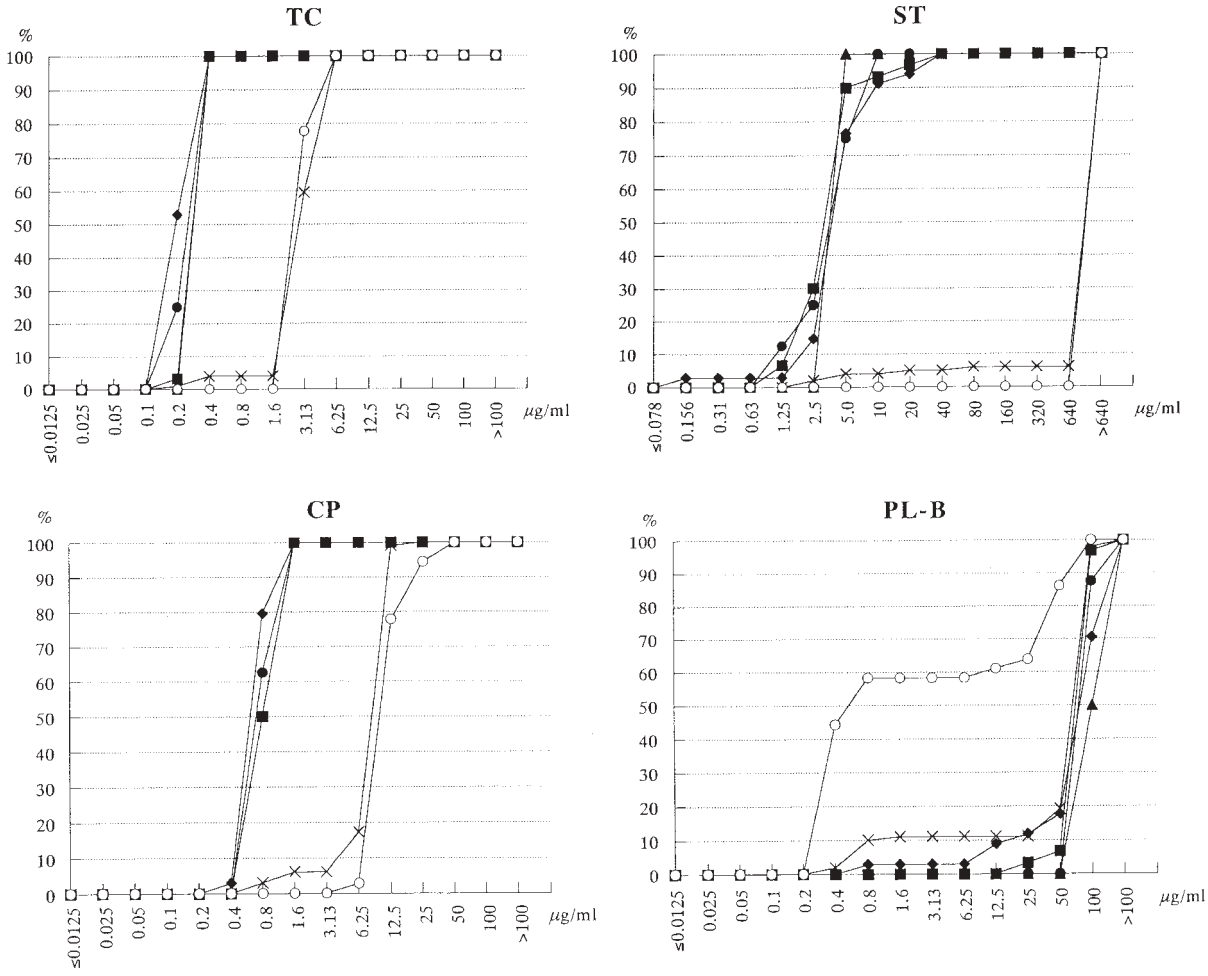
tests, and inoculated by stabbing in test tubes containing a nutrient agar diluted 2 fold with distilled water, after which they were stored at room temperature until use. The number of strains examined was 209 in total, comprising 8 in 1993, 30 in 1994, 34 in 1995, 2 in 1996, 99 in 1998, and 36 in 1999.

Biotyping

Because of the appearance of polymyxin B sensitive *V. cholerae* Ol, definitive biotyping was required. Biotype of the isolated *V. cholerae* O1 strains was determined according to the original criterion, *ie* the production of El Tor hemolysin. The organisms were inoculated on blood agar plates using human and sheep erythrocytes supplemented with and without anti-El Tor hemolysin rabbit serum at the final concentration of 1:20, followed by incubation at 37ºC for 24 hours. The anti-serum was previously prepared in our laboratory.

Susceptibility tests

Minimum inhibitory concentrations (MIC) of the following 8 drugs–ampicillin (Meiji), tetracycline (Nakarai), chloramphenicol (Wako), erythromycin (Dainihon), ofloxacin (Daiichi), nalidixic acid (Wako), sulfamethoxazol-trimethoprim (sulfamethoxazol: Wako, trimethoprim: Sigma) and polymyxin B (Feizer)-



TC: tetracycline, CP: chloramphenicol, ST: sulfamethoxazol-trimethoprim, PL-B: polymyxin B.
—●—1993, —■—1994, —◆—1995, —▲—1996, —×—1998, —○—1999

were determined by multi-point inoculation onto a series of heart infusion agar plates containing doubling dilutions of the drugs from 100 μg/ml to 0.0125 μg/ml, except for sulfamethoxazol-trimethoprim. The mixture ratio of sulfamethoxazol to trimethoprim was 19 to 1, and the concentration of the drug was expressed as the total amount of sulfamethoxazol-trimethoprim. The dilution series from 640 μg/ml to 0.078 μg/ml of drug combination in agar plates (608 μg/ml to 0.74 μg/ml of sulfamethoxazol and 32 μg/ml to 0.0039 μg/ml of trimethoprim) were prepared. A 10-fold dilution of overnight broth culture was inoculated onto each plate using Micro-planter (Sakuma Co model MITP #00257), and incubated at 37ºC for 24 hours. The susceptibility was expressed as MIC of each drug.

RESULTS

Bacterial strains used were typical *V. cholerae* O1 as examined by the routine laboratory methods, and they produced β-hemolysis when cultured on sheep and human blood agar plates. These hemolysis were completely inhibited by addition of anti-El Tor hemolysin serum.

Drug susceptibilities of *V. cholerae* O1 isolates before and after 1997 were quite different except for ampicillin, erythromycin, nalidixic acid and ofloxacin. The MICs of these 4 drugs remained within the expected range until 1999 (Fig 1; EM, ABPC, NA, OFLX).

The susceptibility to tetracycline had been constant with the MICs of 0.2 or 0.4 μg/ml until 1996. However, 95 out of 99 isolates in 1998 and all isolates in 1999 were moderately resistant to tetracycline with its MIC of 3.13 or 6.25 μg/ml (this MIC was about 16 times higher than that examined before 1996). The change of susceptibility to chrolamphenicol was similar to that of tetracycline (Fig l; TC, CP). While 93 out of 99 isolates in 1998 and all isolates in 1999 became highly resistant to sulfamethoxazol-trimethoprim, the MIC went up to about 256 times of the MIC before 1996 (Fig 1; ST). One isolate out of 34 examined in 1996 revealed polymyxin B sensitive, and there were no cholera patients in 1997. When cholera resurfaced during 1998, 11 isolates out of 99 (11%) were found to be sensitive to polymyxin B with the same MIC level to classic cholera vibrios (0.39~0.78 μg/ml) as shown by the cross marked line in Fig 1; PL-B. In 1999, the isolation rate of polymyxin B sensitive strains went up to 58% (21 out of 36 isolates examined) as shown by the open circle line in Fig 1; PL-B.

DISCUSSION

This study provided further support that there was no drug resistant *V. cholerae* O1 in Lao PDR until 1996, but the resistant strains appeared and increased from 1998. Furthermore, the level of resistance to tetracycline and chloramphenicol was not as high as previously reported (Mhalu *et al*, 1979; Glass *et al*, 1980; Ehara *et al*, 1984), yet the strains were highly resistant to sulfamethoxazol-trimethoprim, and interestingly, more than 50% of the isolates (*V. cholerae* O1 El Tor) in 1999 were found to be sensitive to polymyxin B. The sensitivity l1 *V. cholerae* O1 to polymyxin B is decided by growth or failure to grow at a drug concentration of 15 μg/ml. Actually, however, the MIC against El Tor strain was mostly higher than 100 μg/ml and it was lower than 1 μg/ml against the classical strain. From this viewpoint, polymyxin B is no longer useful to differentiate the biotype of *V. cholerae* O1 in Lao PDR.

Previously reported tetracycline resistant *V. cholerae* O1 strains isolated during a cholera epidemic were highly resistant to tetracycline as well as to the other drugs and the resistance was mediated by plasmid, but strains in the present study were moderately resistant to tetracycline and chloramphenicol, and no plasmid was detected as examined in 1998 (Iwanaga *et al*, 2000). In the present study of cholera due to the moderately organisms resistant to tetracycline, clinical data concerned with prognosis of the patients treated with ordinary doses of tetracycline was not available, but the diarrhea supposedly lasted longer than the diarrhea due to sensitive organisms. Besides, if tetracycline is continuously used, there is a possibility that highly resistant clones would be selected. Only one report of cholera epidemic due to polymyxin B sensitive El Tor vibrios was seen in 1974 (Gugnani and Pal, 1974), but thereafter, a polymyxin B sensitive El Tor strain has not been reported as far as the authors are aware.

The cholera vibrio associated with the 7th cholera pandemic was phenotypically hemolytic, therefore, it was designated biotype El Tor (Feeley, 1966). All isolates in the present study were regarded as El Tor biotype because they were hemolytic on blood agar plates using human and sheep erythrocytes, and the hemolysis was completely inhibited by adding anti-El Tor hemolysin serum. In addition to the hemolytic property, *V. cholerae* O1 biotype El Tor is characterized by polymyxin B resistance, cholera phage IV resistance, Voges-Proskauer reaction positive, and agglutination of chicken erythrocytes when the organisms are cultured on nutrient agar plates (Barua and Mukerjee, 1965). Among these biological tests, sensitivity to polymyxin B and cholera phage IV have been recognized to be the most reliable. Therefore, if necessary,

the differentiation of biotypes is usually carried out by examining the susceptibility to polymyxin B in the clinical laboratory, by using disks containing 50 units of polymyxin B. Now, however, we have to bear in mind that the susceptibility to polymyxin B is no longer reliable to distinguish biotypes of *V. cholerae* O1. Polymyxin B sensitive El Tor strains appeared in the epidemic of 1998 with the isolation frequency of 11%, increasing to 58 % in 1999. We are not aware whether this phenomenon is observed only in Lao PDR or elsewhere. In the other countries with cholera epidemic, susceptibility of the organisms to polymyxin B should be monitored. If polymyxin B sensitive El Tor strains are widely distributed in the world, biotype classification should be dependent on the hemolytic properties as originally described. However, the hemolytic property of the isolates was reduced or disappeared soon after the beginning of 7th cholera pandemic, as examined by standard method (Feeley and Pittman, 1963). Therefore, the blood agar method is recommended. The appearance of polymyxin B sensitive El Tor vibrios may bring about a confusion in biotype classification of *V. cholerae* O1.

ACKNOWLEDGEMENTS

This research was supported in part by the Ohyama Health Foundation, Inc.

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