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Original Article

Cloning of Multidrug-Resistant Genes from *Vibrio cholerae* Non-O1 NCTC4716

Anowara Begum*, Teruo Kuroda and Tomofusa Tsuchiya

Department of Molecular Microbiology, Graduate School of Medicine, Dentistry & Pharmaceutical Sciences, Okayama University, Okayama 700 8530, Japan

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Both clinical and environmental multidrug-resistant strains of *Vibrio cholerae* (O1 and non-O1) have been reported from different parts of the world. In this study, we have cloned twenty-five genes associated drug resistance in *Vibrio cholerae* non-O1 NCTC4716 using shotgun cloning method. All of the cloned genes were introduced and expressed in a drug hypersusceptible host *Escherichia coli* KAM32. We observed elevated minimum inhibitory concentration (MIC) of various antimicrobial agents such as fluoroquinolones, aminoglycosides, ethidium bromide and others in all transformants.

Keywords: *Vibrio cholerae*, Drug resistance, Drug-resistant genes, Cloning, Minimum inhibitory concentration (MIC)

Introduction

The incidence of drug resistance is increasing at an alarming rate and causes serious problems in the treatment of infectious diseases against multidrug-resistant (MDR) bacteria[1]. In our country, *Vibrio cholerae* has been recognized as a severe diarrhoeal disease-causing organism in human. Cholera is an emerging and re-emerging infectious disease and specially spread out in many developing countries. Already, a number of clinically isolated multi drug resistant *Vibrio cholerae* O1 and non-O1 has been reported[2-3]. Even though, some published data reported that the presence of antibiotic resistance genes in plasmids or integrons of *Vibrio cholerae,* which are responsible for drug resistance and in many cases the mechanisms of drug resistance are unknown[4].

Many bacteria both Gram-positive and Gram-negative are recently known to be resistance to various antibiotics. This resistance property is either plasmid-mediated or present on the chromosome[4]. Several drug resistant patterns are known to be at present. They include (a) inactivation of drug due to degradation or modification, (b) change of drug targets, (c) production of a bypass for the target pathway, (d) active drug efflux from cells, and (e) changes of membrane permeability especially in Gram-negative bacteria[5]. Due to the different drug-resistant patterns, nowadays treatment of patients infected with various organisms has become a serious problem. Detail studies to understand the mechanisms of drug resistant are more important to develop a new drug and treatment of patients. So, it is more significant to cloning of the new drug-resistant genes and characterized their properties that are responsible for drug resistance of *Vibrio cholerae* non-O1.

Materials and Methods

Bacteria and growth

V. cholerae non-O1 and *E. coli* KAM32 (Δ*acr*AB, Δ*ydh*E)[6], a derivative of TG1, were used in this study. *V. cholerae* non-O1 were grown in Luria-Bertani (LB) medium (containing 1% polypeptone, 0.5 % yeast extract, 1.0% NaCl and 1.5% agar, pH 7.0), and *E. coli* cells were grown in L medium (containing 1% polypetone, 0.5% yeast extract and 0.5% NaCl, pH 7.0) at 37°C with shaking. The growth was measured by optical density at 650 nm[5].

Gene cloning and transformation

Chromosomal DNA of *V. cholerae* non-O1 was extracted by the method of Berns and Thomas[7]. The DNA was partially digested with *Sau*3AI, and the fragments of 4 to 10 kb were separated by sucrose density gradient centrifugation. Plasmid pBR322 was prepared by QIAGEN according to the manufacturer instruction. Plasmid pBR322 was digested with *Bam*HI and dephosphorylated with shrimp alkaline phosphatase and then ligated with chromosomal DNA fragment of *V. cholerae* non-O1 by using ligation kit (Version 2, Takara, Japan). Competent cell of *E. coli* KAM32 were transformed

Corresponding author:
Dr. Anowara Begum, Associate Professor, Department of Microbiology, University of Dhaka, Dhaka 1000, Bangladesh
Tel (Office): (02) 9661920-73/7747; Tel (Home): (02) 9121737; Fax: +880 (02) 8615583; E-mail: anowara71@yahoo.com

with the recombinant plasmids and were spread on LB agar plates supplemented with ampicillin (60 μg/ml), acriflavin (10 μg/ml), 4,6-diamidino-2-phenyl indole (DAPI, 0.5 μg/ml), ethidium bromide (EtBr, 8 μg/ml), norfloxacin (0.005 μg/ml), rhodamin 6G (R6G, 8 μg/ml), tetraphenyl phosphonium chloride (TPPCl, 8 μg/ml), and streptomycin (8 μg/ml). The plates were incubated at 37°C for 24 h. Plasmids from all candidates were retransformed into *E. coli* KAM32, and then spread onto the respective drug containing plates.

Sequencing

Plasmids were extracted from retransformed candidates for DNA sequencing. The nucleotide sequence was determined by the dideoxy chain termination method using an automated DNA sequencer[8] (ALF Express, Pharmacia Biotech, USA).

Determination of minimum inhibitor concentrations (MICs)

The MICs of various drugs were determined in Muller-Hinton broth (Difco, USA) containing different drugs at various concentrations by two-fold dilution method[9]. Cells in the test medium were incubated at 37°C for 24 h. The MICs were determined as the lowest concentration of antimicrobial agents at which bacterial cells were unable to grow.

Drug susceptibility test

Drug resistance pattern were determined by streaking a single colony of each candidate on LB agar supplemented with 60 μg/ml ampicillin, 10 μg/ml acriflavin, 0.5 μg/ml 4,6-diamidino-2-phenyl indole (DAPI), 8 μg/ml ethidium bromide (EtBr), 0.005 μg/ml norfloxacin, 8 μg/ml rhodamin 6G (R6G), 8 μg/ml tetraphenyl phosphonium chloride (TPPCl), and 8 μg/ml streptomycin. All plates were incubated at 37°C for 24 h[5].

Results and Discussions

Drug-resistant pattern of V. cholerae non-O1

For selection of drugs and cloning of drug-resistant gene(s), at first we determined the minimum inhibitory concentrations (MIC) of *V. cholerae* non-O1 (Table 1) and also determined the MICs of *E. coli* KAM32, a drug hypersusceptible strain. It was observed that *V. cholerae* non-O1 showed higher resistance to TPPCl, EtBr, R6G, streptomycin, DAPI, norfloxacin and acriflavin as compared to *E. coli* KAM32. For this reason, we selected these drugs for selection of cloned drug resistant genes. The results shown in Table 1 also suggest that there might be some drug-resistant gene(s) present in *V. cholerae* non-O1 NCTC4716 that might be responsible for resistance against TPPCl, EtBr, R6G, DAPI, acriflavin, norfloxacin and streptomycin.

Table 1. *Minimum inhibitory concentrations (MICs) of Vibrio cholerae non-O1 NCTC4716 and Escherichia coli KAM32*

Drug	Minimum inhibitory concentration (μg/ml)	
	V. cholerae non-O1	*E. coli* KAM32
DAPI	4	1
TPPCl	128	4
Acriflavin hydrochloride	4	1
Ethidium bromide	16	4
Rhodamin 6G	64	4
Carbicillin sodium	2	2
Ampicillin sodium	2	2
Kanamycin sulphate	4	1
Streptomycin sulphate	16	1
Sodium deoxycholate	512	1,024
SDS	512	256
Chloramphenicol	1	1
Tetracycline hydrochloride	4	1
Norfloxacin	0.06	0.03
Erythromycin	4	2

DAPI = 4,6-Diamidino-2-phenyl indole; TPPCl = Tetraphenyl phosphonium chloride; SDS = Sodium deodocyl sulphate

Cloning of drug resistant genes and drug resistant pattern

In an attempt to cloning of drug-resistant genes from *Vibrio cholerae* non-O1 due to drug efflux pump/membrane transporter, or any other mechanisms we used shotgun cloning methods and transformed all the recombinants plasmids into *E. coli* KAM32, a drug hypersusceptible host. We found 25 candidate clones that harbour recombinant plasmids using various concentrations of different antimicrobial agents. One clone was obtained from acriflavin plate, two from DAPI, 14 from EtBr, one from norfloxacin, one from R6G and six from TPPCl (Table 2). The clones that were isolated from acriflavin, DAPI, EtBr (except E-1) and TPPCl plates were all resistant to acriflavin, DAPI, EtBr and TPPCl. Clones of E-1 showed resistant not only to EtBr but also to DAPI, norfloxacin and TPPCl. The norfloxacin-resistant clone also showed resistant to DAPI. The clone that was isolated from R6G was not only resistant to R6G but also showed resistant to EtBr and TPPCl. The restriction pattern for all of the plasmids had been checked. Restriction pattern conferred that the same drug specific plasmids possessed common DNA fragment. These data suggested that these cloned genes might be major cause of drug resistance in *V. cholerae* non-O1.

Table 2. *Drug resistance pattern of different transformants*

Drug used for selection (μg/ml)	Candidate	Drug used for sensitivity test (μg/ml)						
		Acriflavin (10)	DAPI (0.5)	EtBr (8)	Norfloxacin (0.05)	R6G (8)	TPPCl (8)	Streptomycin (8)
Acriflavin (10)	A-1	+	+	+	-	-	+	-
DAPI (0.5)	D-1	+	+	+	-	-	+	-
	D-2	+	+	+	-	-	+	-
EtBr (8)	E-1	-	+	+	+	-	+	-
	E-2	+	+	+	-	-	+	-
	E-3	+	+	+	-	-	+	-
	E-4	+	+	+	-	-	+	-
	E-5	+	+	+	-	-	+	-
	E-6	+	+	+	-	-	+	-
	E-7	+	+	+	-	-	+	-
	E-8	+	+	+	-	-	+	-
	E-9	+	+	+	-	-	+	-
	E-10	+	+	+	-	-	+	-
	E-11	+	+	+	-	-	+	-
	E-12	+	+	+	-	-	+	-
	E-13	+	+	+	-	-	+	-
	E-14	+	+	+	-	-	+	-
Norfloxacin (0.005)	N-1	-	+	-	+	-	-	-
R6G (8)	R-1	-	-	+	-	+	+	-
TPPCl (8)	T-1	+	+	+	-	-	+	-
	T-5	+	+	+	-	-	+	-
	T-6	+	+	+	-	-	+	-
	T-7	+	+	+	-	-	+	-
	T-8	+	+	+	-	-	+	-
	T-9	+	+	+	-	-	+	-

DAPI = 4,6-Diamidino-2-phenyl indole; EtBr = Ethidium bromide; R6G = Rhodamine 6G; TPPCl = Tetraphenyl phosphonium chloride

Partial sequencing

Partial DNA sequencing of newly cloned genes was performed to compare the sequencing data with several published[3,10-11] sequences of drug-resistant genes like *vcm*A, *vcr*M and *vcm*B. Comparison of deduced amino acid sequence between *V. cholerae* ElTor N16961 and new clones of *V. cholerae* non-O1 showed that some of them (ethidium bromide, DAPI, TPPCl and Acriflavin candidates) were belonged to drug efflux pumps/membrane transporter. R-1 cloned showed similarity with a regulatory protein that regulates some drug-resistant genes that is present in *V. cholerae* non-O1.

This study concluded that there might be some drug-resistant genes present in chromosome of *V. cholerae* that are one of the major causes of drug resistance in vibrios.

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