

Chapter 2

Asiatic Cholera: Mole Hills and Mountains

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Abstract The disease cholera has persisted in Asia since time immemorial. Almost all the pandemic phases of cholera had its origin from the Indian subcontinent. Historically, waves of cholera have wiped many million lives in this region mainly due to the general insanitary conditions and poor management of the disease. All the three cholera causing vibrios namely classical, El Tor, and the O139 have emerged from Asia at different times and one was replaced by the other by overcoming the acquired immunity. Antimicrobial resistance was not a big problem in the early 1960s as its use was very limited. With the use of third-generation drugs, *Vibrio cholerae* has acquired many resistance mechanisms over the passage of time and also due to prevailing antibiotic pressure. With its biotypes/serotypes there are considerable variations at the genetic level and many clones of *V. cholerae* have been detected. Recently, the hybrid strain of El Tor has spread in many Asian countries causing several cholera outbreaks. However, the importance of such genetic changes was not fully strengthened in epidemiological perspective. The perspectives of cholera vaccines have shown to be encouraging in many recent vaccine trials in Asia. Traditional medicine has lost its glory as it lacks the scientific evidence in curing infectious diseases. Some of the herbal formulations are now reconsidered for extensive research. The control measures for preventing cholera are yet to gain momentum in many Asian countries, as it involves coordination of government and the public with adequate funds to revamp the water supply and waste disposal systems. On the other hand, the clinical management of cholera and other diarrheal diseases are largely under the control in Asia.

2.1 Introduction

The Asian continent has been considered as the cradle of cholera for many centuries. In many publications it was shown that the high temperature, relative humidity, and

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intermittent rain fall form ideal climatic conditions for the incidence of cholera. Recently, this hypothesis was further strengthened with oceanographic studies, which has shown that there is a strong correlation between El Niño and the incidence of cholera. The classical, El Tor biotypes as well as the O139 serogroup had emerged from the Asian region. Cholera is still a problem in Asia, as progress toward standard of good living with all the public health facilities including uninterrupted supply of safe drinking water, environmental sanitation, implementation of efficient vaccine are somehow long-winded. The emergence and reemergence of different phenotypic and genetic variants of *Vibrio cholerae*, the causative organism of cholera, show its ability for survival in the environment and cause infection in the human host. This chapter reviews the current status of cholera in many Asian countries and many aspects on the causative organism *V. cholerae* including antimicrobial resistance and molecular epidemiology.

2.2 Cholera in the Indian Subcontinent

Cholera is an ancient disease in Indian subcontinent. Historically, it was believed that the first six classical cholera pandemics originated from the Indian subcontinent [1]. In India, the classical biotype was replaced by the El Tor from 1965 [2]. In some areas in India such as Raipur, the classical cholera prevailed till 1970 and the subsequent cholera outbreaks in 1975, 1977, 1979–1981 were caused by El Tor vibrios [3]. Continuous monitoring of cholera epidemics helped to detect frequent changes in the incidence of *V. cholerae* serogroups. Younger age group (<15 years) was the most affected population in the 1988 cholera outbreak in Delhi [4]. In Sevagram, Maharashtra, the incidence of O1 serotype Ogawa was predominant with intermittent appearance of O139 serogroup during 1992, 1997 [5]. The incidence rate of O1 and O139 serogroups in Delhi during 1992, 2000 was 81 and 14%, respectively [6]. Occurrence of O139 serogroup in Delhi was low between 1994 and 1999, but reemerged during 2000. A questionnaire-based survey conducted to estimate the water-borne infection with the use of Ganges River indicated the incidence of cholera (33 cases) among families who used the river water for many purposes including washing clothing and bathing [7]. During 2002 cholera outbreak in Chandigarh, *V. cholerae* O1 Ogawa was isolated from 18% of the hand-pump water samples [8].

The annual incidence of cholera estimated using the population census as the denominator and the age-specific number of cases as numerator showed comparatively low in Jakarta, Indonesia (0.5/1,000), than in Kolkata, India (1.6/1,000), and Beira, Mozambique (4.0/1,000) [9]. In this study, children below 5 years of age were found to be the most vulnerable group for cholera infection. *V. cholerae* O1 and O139 consecutively appeared during cholera outbreaks (2002–2003) near Karachi [10]. This study has also revealed that children less than 2 years of age were the most affected age group with O1 (49%) than O139 (21%). Remote areas such as Andaman and Nicobar Islands were free from cholera for many years. The first

cholera outbreak was recorded during early 2000s due to the spread of *V. cholerae* O1 from the main land [11]. In 2002, cholera was identified due to El Tor vibrios among Nicobarese tribe in 16 villages with an attack rate of 12.8% and a case fatality ratio of 1.3% [12]. Concomitant infections by *V. cholerae* O1 and O139 serogroups were reported in 2000 from a large cholera outbreak in Ahmedabad, India [13]. In Delhi, the serotype switchover from Ogawa to Inaba has started in 2004 and 88% of the strains were identified as Inaba during 2005 [14]. Among children below 5 years of age, the incidence of cholera in Delhi was 33%. Cholera caused by the Inaba serotype was also reported from other parts of India such as Kolkata, Orissa, Andaman and Nicobar Islands [15–17].

In Bangladesh, the classical biotype was replaced by El Tor vibrios during 1964–1973 [18, 19]. During 1973–1979, cholera due to classical biotype was not detected in Matlab and Dhaka, but was predominant (79%) in southern regions of Bangladesh during 1988–1989 [20]. Intermittent appearance of classical cholera was recorded during 1979 to 1981 [21]. Classical cholera appeared in the form of large epidemic starting from Matlab, Comilla, and Dhaka during late 1982 and spread to other districts replacing the El Tor biotype [19, 21]. Phenotypically, the new classical strains were identical to the one that prevailed a decade earlier and the virulence features and seasonality resembled to that of El Tor strains prevailing at that time. It was hypothesized that the classical strains of *V. cholerae* O1 were indigenous to Bangladesh [22]. A 33-year (1966–1988) data analysis provided much information from Bangladesh [23]. Between 1966 and 1988, both classical and El Tor biotypes were prevailed and by 1988, the classical biotype disappeared. The serotype prevalence during 1988–1989 was also interesting as El Tor belonged to Inaba, whereas the classical strains to Ogawa type.

Studies conducted from 1985 to 1991 in Bangladesh indicated that the incidence of cholera was among children below 5 years (24%) and children below 2 years of age accounted for 10% of the cases [24]. The overall case fatality during epidemics was 4.0%. A mathematical Ogawa–Inaba model based on the 40 years (1966–2005) analysis explained the serotype changes in cholera case patterns in Bangladesh when the cross-immunity to one specific serotype was high [25]. It was hypothesized that intermittent appearance of Inaba serotype might be related to its long-term immunity against Ogawa.

Based on the spatial patterns and exploratory spatial data analysis, the risk factors for cholera were associated with environmental niches [26]. Environmental studies conducted during 2004 in Mathbaria and Matlab, Bangladesh, revealed that both *V. cholerae* O1 and O139 serogroups occurred predominantly as viable but non-culturable state [27]. However, culturable cells were also detected in the biofilms, which were considered as additional reservoirs of toxigenic *V. cholerae* in the aquatic environments during inter-epidemic seasons. Isolation of *V. cholerae* O1 from the aquatic environments of Bangladesh through selective enrichment using antibiotics has reemphasized the hypothesis that the humans act as reservoirs of this pathogen during inter-epidemic periods and spreading occurs through contaminated water [28].

2.3 Other Asian Countries

In Japan, cholera epidemics during 1882 and 1895 were recorded in Fukushima, northeastern part of Japan [29]. Due to cholera epidemics, the health policy has changed in Japan during Meiji period (1868–1912) [30, 31]. Changes in the cholera diffusion pattern during this period demonstrate improvements in the transportation and most importantly, growth in socio-economic systems. In Okinawa, Japan, the incidence of cholera was high in 1879 [32]. Laboratory data with *V. cholerae* O1 strains collected between 1977 and 1987 revealed low number of domestic cholera in Japan [33]. Studies conducted in various environments of Japan and imported seafood showed prevalence of non-toxigenic *V. cholerae*. In the Port of Osaka, Japan, *V. cholerae* O1 was detected during 1987–2001 and all the strains were closely related and non-toxigenic [34].

Contaminated seafood is associated with cholera in many South Asian countries. In Hong Kong, a local cholera outbreak was related to the consumption of shellfish that were kept live in contaminated seawater [35]. The cholera toxin (CT)-producing *V. cholerae* non-O1 strains were isolated from seafood in Taiwan [36]. Turtles and their breeding environment are the major reservoirs of *V. cholerae* and responsible for many outbreaks of cholera in Sichuan Province and Guangzhou area, China during 2003–2005 [37, 38]. In an investigation it was shown that turtles and other seafood harbored toxigenic *V. cholerae* O139 [39]. In Zhejiang Province, the incidence of O1 serogroup of *V. cholerae* was found to be high (9%) in turtles and cholera epidemics in this region might be associated with consumption of contaminated turtles [40].

Surveys conducted in eastern region of Saudi Arabia, mainly with expatriate workers and household contacts with *V. cholerae* infection, showed the prevalence of *V. cholerae* O1 El Tor serotype Ogawa in 113 diarrheal patients (6.0 per 100,000 population per year), 28 asymptomatic cases, and 16 of 982 household contacts of index patients [41]. Interestingly, the O1 strains isolated from asymptomatic cases were non-toxigenic as detected by CT-ELISA. Following the Iraq war, the communicable disease control program was disturbed, resulting in cholera epidemics in several districts of Basrah, Iraq, in 2003 [42]. A 6-year study (1997–2002) conducted in hospitals in Zabol city, Iran, indicated the incidence of cholera due to *V. cholerae* O1 Ogawa was 10% with maximal number of cases in 1997 (22%) [43]. Among the infected cases, there were no differences in age or social and economic strata. Almost 19% of the cases in this study were from neighboring Afghanistan. Cholera outbreak struck Kabul, Afghanistan, in 2005 and spread nationwide. The health authorities gave importance to the disease control program that included proper management and treatment supported by partner agencies that kept the mortality rate well below 0.1% [44]. Processed and raw foods imported from Asian countries have also contributed to the incidence of cholera in non-endemic areas. Three cases of cholera reported in Australia in 2006 had a link with consumption of raw whitebait imported from Indonesia [45]. In France, a total of 129 imported cases of cholera were recorded during 1973–2005 [46]. During 1980s, 94% of the patients were infected in Morocco and Algeria, and during the rest of

the period, cholera was associated with patients visiting Asia and other African countries.

Since 1873, cholera was common in Sarawak, Malaysia, and the classical biotype was common prior to 1961 [47]. In Vietnam, incidence of cholera has distinct temporal and seasonal trends. Between 1991 and 2001, cholera mostly affected the central coast during May to November without having any climatic association with eight recorded outbreaks [48]. However, studies conducted from 1996 to 2002 in Bangladesh indicated that weather factors could play a role in the epidemiology of cholera with either increase or decrease during monsoon [49]. Based on a study conducted during 1993–1999, the epidemic and sporadic cholera occurs in the western regions of Indonesia during low rainfall, whereas in eastern parts, heavy rainfall has contributed to the cholera transmission [50]. A 6-year hospital-based study on the incidence of El Tor cholera in Thailand indicated that the affected age group ranged from 2 months to 15 years with an average age of 3 years [51].

2.4 Association Between *V. cholerae* and Parasites

Incidence of *V. cholerae* in association with enteric parasites has been reported in many cholera-endemic regions. In Nepal, the incidence of cholera is associated with co-infection with enteric parasites such as *Giardia lamblia* and *Ascaris lumbricoides* [52]. In a recent study, it was shown that the co-infection with geohelminth parasites reduces the magnitude of the cholera vaccine response [53]. Among Indian children, the infection due to cholera and parasites, especially with *G. lamblia*, seems common [54]. The incidence of mixed infection with *V. cholerae* O1 and *A. lumbricoides* and *G. lamblia* was more than *Trichuris trichiura* and *Entamoeba histolytica* in Kolkata [55]. This investigation also showed that the combined infection of *V. cholerae* and parasites was common in children aged between 2 and 10 years. The association between parasites and *V. cholerae* is not clear, but seems advantageous for both the pathogens. In a rat colon model, it was proved that *E. histolytica* trophozoites and cholera toxin enhanced secretion of mucin glycoproteins and stimulated colonic glycoprotein synthesis [56].

2.5 The O139 Cholera

A novel non-O1 strain of *V. cholerae* was first discovered in 1993 that has caused large epidemic in Madras, southern part of India [57]. This strain of *V. cholerae* not agglutinated with antisera from O1 to O138 was included as a new serogroup O139 [58]. This serogroup had spread quickly to Bangladesh and to other states of India within a span of about 10 months [59, 60]. Since the *V. cholerae* O139 was first discovered in the areas surrounding the Bay of Bengal (Tamil Nadu, Andhra Pradesh, West Bengal and Bangladesh), this serogroup has a synonym “Bengal.” In a span of 1 year, this serogroup has been reported in many Asian countries (Fig. 2.1). The O139 infection produced severe dehydrating diarrhea, which is indistinguishable

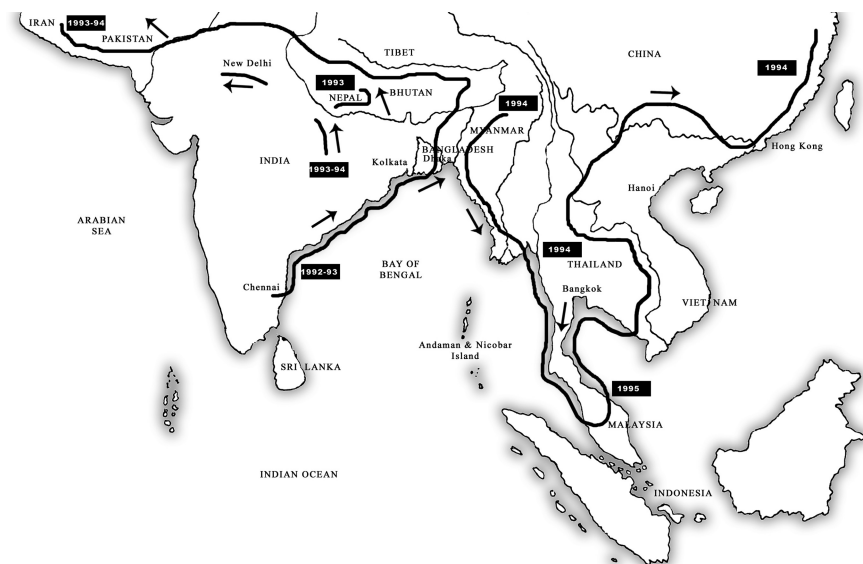


Fig. 2.1 Spread of *Vibrio cholerae* O139 in Asia during 1993–1995

from clinical cholera and does not appear to confer any cross-protection from the O1 serogroup [61]. Based on the clinical symptoms and severity of diarrhea, the disease caused by *V. cholerae* O139 is now considered as cholera [62] and the infections caused by the rest of the non-O1 and non-O139 serogroups are known as “cholera-like diarrhea.”

Before its total replacement, the O139 prevailed along with the O1 serogroup in many countries. The affected age group by the O139 infection depends on the period and place of its occurrence. In Delhi the incidence of O139 and O1 cholera was frequent in children below 5 years of age [63]. In this study, the incidence of both the serogroups was detected among 1.4% of the children with cholera. A similar epidemiological observation was made in Karachi, Pakistan [64]. The incidence of O139 among children during 2003 in Pakistan was 21% and the infected patients were more likely to be febrile ($P < 0.001$) [10].

After its initial explosive epidemic during late 1992 and early 1993, occurrence of O139 serogroup had declined in many cholera-endemic regions [65, 66]. Between 1997 and 2000, incidence of cholera due to O139 serogroup decreased to 3.8% in rural Bangladesh [67]. Resurgence of O139 cholera was reported in many Asian countries including Pakistan (2000–2001), India (1997, 2001), and Bangladesh (2002), mostly affecting the older age groups [68–72]. From 1999 to 2000, most of the cholera outbreaks in India were caused by the O139 serogroup [73]. Investigations conducted in Indonesia revealed that the O139 serogroup had not invaded into this country till 1999 [50]. The first incidence of O139 was recorded in Baghdad, Iraq, in 1999, though the numbers of cases were less [74].

Imported cases of O139 cholera were reported soon after its emergence in Asia in California [75] and other parts of the USA [76], Japan [77], and Denmark [78]. The recurrent infection caused alternatively by the O1 and O139 serogroups in cholera-endemic regions emphasize the fact that the role of acquired immunity plays an important role in the emergence and dissemination of specific serogroup in a population. In addition, rapid genetic reassortment in *V. cholerae* O1 and O139 serogroups might play a role in the changing epidemiology of cholera [79]. It is still a mystery that why the so-called highly infectious O139 serogroup has not spread to the other cholera-endemic regions such as Africa.

2.6 Antimicrobial Resistance

Resistance of *V. cholerae* to antimicrobials used for the treatment of cholera is not uniform in many countries. The emergence of resistance is mainly due to the prevailing antibiotic pressure caused by overuse of antimicrobials. In several findings, it was proved that the susceptibility pattern of *V. cholerae* to the antimicrobials is constantly changing over a period of time. In India, *V. cholerae* isolated during mid-1970s were susceptible to trimethoprim–sulfamethoxazole and patients who received treatment with this drug recovered quickly [80].

In the early 1990s, *V. cholerae* O1 El Tor were susceptible to many antimicrobials including nalidixic acid, co-trimoxazole, chloramphenicol, and streptomycin in India [81]. The classical as well as El Tor strains prevailed in Bangladesh during 1988–1989. However, tetracycline resistance was detected only with the classical strains, whereas the El Tor strains remained susceptible to this drug [20].

Based on the antimicrobial resistance profiles and the resistance gene composition, *V. cholerae* O1 strains isolated during cholera outbreaks in Vietnam were described as different clones [82]. Unlike in many African countries, tetracycline-resistant strains are not common in the Asian region. However, intermittent appearance of tetracycline resistance in *V. cholerae* O1 has been reported from several Asian countries [83–86]. Cholera patients infected with tetracycline-resistant strains of *V. cholerae* purged longer with greater stool volume while receiving treatment with this drug [83].

In southern Thailand, an unusually large epidemic of cholera due to *V. cholerae* O1 was recorded during 1997–1998 [85]. All the strains were resistant to tetracycline and belong to a unique clone and this trend has not been reported in Thailand since 1993. The El Tor vibrios are resistant to polymyxin B, which is also a biotypic marker to differentiate them from classical vibrios. The El Tor strains (11%) isolated from an outbreak of cholera in Laos during 1998 was susceptible for polymyxin B and resistant to tetracycline and sulfamethoxazole–trimethoprim, which were different from those of previous strains [87]. In the following year, the incidence of polymyxin B susceptibility increased to 58%. The Kelantan (Malaysia) cholera epidemic in 1998 was identified due to tetracycline-resistant strains [84]. To control the outbreak in this region, erythromycin was substituted for tetracycline. Tetracycline resistance was uncommon in India for many years. However, 27 and

15% of Ogawa and Inaba strains from Kolkata were, respectively, resistant to this drug during 2005 [86]. A recent study conducted in Thailand showed that most of the *V. cholerae* strains isolated from cholera patients were susceptible to ceftriaxone and quinolones, which were used in the treatment of cholera [51]. However, in neighboring countries this scenario is completely different, as quinolone resistance in *V. cholerae* was common for many years.

V. cholerae O139 strains that emerged during early 1990s in several Asian countries were resistant to trimethoprim, sulfamethoxazole, and streptomycin, similar to the El Tor vibrios. However, the reemerged O139 strains were susceptible to these antimicrobials [68, 72, 88, 89]. Possible deletion of a 3.6 kb region of the SXT element in the reappeared O139 strains during 1995 in Bangladesh was thought to be responsible for susceptibility to these antimicrobials [90]. Some of the early O139 isolated in India were resistant to tetracycline, ampicillin, chloramphenicol, kanamycin, and gentamicin and a 200 kb self-transmissible plasmid carried the encoding genes for multidrug resistance [91].

V. cholerae O1 strains with reduced susceptibility to fluoroquinolones have been reported from French travelers returning from India [92]. The trend of reduced susceptibility toward ciprofloxacin seems increasing in certain states of India. About 46% of the Ogawa strains isolated in 2003 showed reduced susceptibility to ciprofloxacin [93]. In Sevagram, India, resistance to tetracycline varied from 2 to 17% [5]. Majority of the Iranian strains were resistant to streptomycin, chloramphenicol, co-trimoxazole, and tetracycline [94]. The newly emerged Inaba strains from Delhi, India, were resistant (96%) to co-trimoxazole, furazolidone, and nalidixic acid [14]. *V. cholerae* O1 appeared during 1995, 2000, and 2002 in Vietnam had different resistance profiles, as they harbored either in the class 1 integron (strains of 1992) or in SXT constin (strains of 2000) [82]. Detailed studies on integrons are covered in Chapter 9.

2.7 Phage Typing of *V. cholerae* O1 and O139

During the pre-molecular era, phage typing of *V. cholerae* O1 was considered as one of the powerful tools in discriminating the strains of epidemiological interest. Four specific phages were used for biotyping of classical *V. cholerae* [95, 96]. Basu and Mukerjee [97] established a systematic grouping of El Tor strains. Under this scheme, a panel of five phages was used for many years. In 1971–1984 cholera outbreaks in Hyderabad, India, phage types T1, T2, and T4 were dominated during classical cholera period and types T2 and T4 dominated among El Tor strains in later years [98]. In due course of time, the classical strains disappeared in India and the El Tor strains were mostly clustered with phage types T2 and T4. Due to this limitation of Basu and Mukerjee's El Tor phages, a new phage typing scheme was introduced with a panel of five additional phages [99, 100]. In this new scheme the phage type numbers were increased to 146 when about 1000 *V. cholerae* O1 El Tor strains were tested. These phages are now being used in the current phage typing of El Tor vibrios at the National Institute of Cholera and Enteric Diseases

(NICED), Kolkata, India. Phage typing scheme for differentiating the *V. cholerae* O139 was also established in NICED with a panel of five lytic phages [101]. A comparative study of phage types in 1993–1994 and 1996–1998 showed higher percentage types T1 (40.5%) and T2 (32.1%). The phage typing scheme is undergoing rapid changes as clustering of certain types needs to be subtyped to find the epidemiological significance of strains.

2.8 Molecular Epidemiology

Molecular epidemiological studies showed frequent emergence of new epidemic clones among O1 and O139 strains isolated from the cholera patients and environmental sources, as reflected in the genomic structure analysis, location of rRNA, and CTX prophages. The detailed outlines in molecular epidemiology of cholera are described in Chapter 7.

A retrospective analysis of *V. cholerae* O1 using RFLP of enterotoxin genes revealed the presence of indigenous and exogenous strains in Hong Kong since 1978 [102]. However, the vibrios isolated during a cholera outbreak among Vietnamese refugees in Hong Kong are indistinguishable, but distinct from the strains isolated in Hong Kong prior to the outbreak [103]. *V. cholerae* O1 strains collected from domestic and imported cases of cholera that occurred between 1984 and 1997 in Aichi prefecture in Japan were subjected to PFGE and the results suggested that a new clone was introduced after 1993 from overseas and disseminated [104]. The Miri Sarawak outbreak during 1997–1998 might be due to multiple *V. cholerae* O1 clones prevailed during this period [105]. *V. cholerae* O1, O139, and rough strains were isolated from seafood samples in Malaysia during 1998–1999 and the PFGE results suggested that the O139 and rough strains would have the origin from Bengal and Thailand–Malaysia–Laos, respectively [106]. *V. cholerae* O1 strains MO1 and MO477 isolated from Madras, India, during 1992–1993 were reported as probable progenitor strains, which are neither truly classical nor El Tor in the different assay including polymyxin B susceptibility (resistant), classical and El Tor phages (resistant), *ctx* RFLP (identical to O139), copy number of *ctx* (identical to O139), and the outer membrane protein profile (identical to O139) [107].

Both toxigenic and non-toxigenic *V. cholerae* O1 were isolated in Taiwan during 1993–1995 from imported cases of cholera and food samples [108]. In this investigation, prevalence of single clone was detected by ribotyping and enterobacterial repetitive intergenic consensus (ERIC) PCR. The sequence information of *ctxB* gene is considered to be one of the useful molecular epidemiological markers based on the specific base substitutions at positions 115 and 203 [109]. In Taiwan, *V. cholerae* O1 strains isolated from imported seafood and sporadic cholera cases had *ctxB* polymorphism of genotype 1, whereas the 1962 strains isolated from cholera epidemic and soft-shelled turtles belonged to genotype 2 [110].

The O139 strains isolated from India, Bangladesh, and Thailand during 1993 were clonally similar as identified by the PFGE [111]. The Asian O139 strains

isolated during 1993–1994 epidemic contained 1–4 copies of *ctx* gene with 55 genotypes as detected in the *ctx* RFLP [112]. The RFLP analysis of *ctx* was found to be superior to ribotyping method for O139 strain discrimination as the later contained only few types. The important molecular event that converted an O1 El Tor strain to O139 serogroup might be due to lateral gene transfer (LGT) event. Prevalence of 64 novel alleles among 51 sequence types from 9 sequenced loci were detected among 96 strains of O139 collected from 1992 to 2000 in Kolkata, India [113]. This study further strengthened the genetic diversity of O139 serogroup during the course of its rapid expansion and recurrent reappearance. The structure, organization, and location of CTX prophages of *V. cholerae* O139 that appeared during different years in Kolkata also revealed the fact that the genomic configuration of the pathogen is very dynamic [89]. The genomic diversity of *V. cholerae* O139 strains that appeared in India and Bangladesh between 1992 and 1998 was tested by using ribotyping and CTX genotyping methods [114]. In this study, six distinct ribotypes were identified, of which B-I–B-V types shared 11 different CTX genotypes (A–K).

The O139 serogroup had a new 35 kb genomic region replacing the O1 somatic antigenic region. Interestingly, this 35 kb region was genetically stable for many years as evidenced from RFLP analysis using many strains collected from South Asia at different periods of time [115]. In addition, the South Asian strains were different from a strain isolated in Argentina indicating the South American strain was unique and had a different origin [115]. The first O139 cholera epidemic appeared in China in 1993 after its first detection in India in 1992. Seven different ribotypes were detected with O139 strains collected in China during 1993–1999 and many strains carried two or more copies of *ctx* gene [116]. Some of the *ctx*-negative O139 strains were grouped into three separate ribotypes [116]. It is evident from this study that the O139 had multiple origins and caused epidemic and sporadic cholera in China. The remerged O139 strains during 2002 in Bangladesh belonged to a ribotype, corresponding to one of the two ribotypes identified during 1993 [72].

V. cholerae O1 El Tor strains isolated before and after the O139 epidemics in India revealed that the strains were different in their genetic profiles based on the RFLP of rRNA genes and CTX genetic element [117]. Till 1997, three different clones of *V. cholerae* O139 prevailed in India, as shown in the *Hind*III digestion patterns with *cxtA* probe [88]. The same molecular trend was also reported from Bangladesh [118]. The ribotype BII of *V. cholerae* O139 was responsible for a cholera outbreak in Ahmedabad in 2000, but their PFGE patterns were different from the strains isolated during 1992–1997 [13]. In the same outbreak, *V. cholerae* O1 was also isolated, but their PFGE profiles matched with clones existed in Kolkata during that time. These findings suggest that there is a continuous genetic reassortment among *V. cholerae*, whenever there is a serotype or serogroup replacement.

The epidemic O1 and O139 strains isolated between 1994 and 2002 are differentiated from non-epidemic strains by PFGE in Hong Kong [119]. Overall, 60 distinct PFGE profiles were obtained in this study, which are different from strains isolated from imported cholera cases, mainly from Indonesia, India, and Pakistan. El Tor vibrios identified from domestic and imported cases of cholera in Japan were

compared by PFGE [120]. The PFGE subtypes *NotI*-AI and *Sfi*-I were found to be widely distributed in Asia.

Based on the ribotyping results, *V. cholerae* O1 Ogawa isolated in 1999–2000 and Inaba strains of 2001–2002 in Thailand exhibited different *Bgl*I profiles confirming the change in the genetic constituent [121]. The ribotyping and PFGE profiles of several outbreak strains of *V. cholerae* O1 Ogawa in metropolitan area of Kuala Lumpur, Malaysia during 1998 matched with those identified in Taiwan, Colombia, and several Asian regions (ribotype V/B21a) and Senegal (ribotype B27) [122]. However, the Inaba strains isolated during the same outbreak exhibited a new ribotype pattern (type A). The O139 strains isolated from surface waters in Malaysia uniformly harbored a 2.0 MDa non-conjugative plasmid and the *ctxA* gene, but were genetically different as determined by RAPD-PCR [123].

The O139 cholera outbreaks are related with consumption of contaminated foods in many parts of China. Outbreaks in Sichuan Province, Jiangxi Province, and Guangzhou area were due to contaminated turtle, as evidenced by PFGE analysis with strains isolated from the patients and suspected food samples [37, 38, 124]. In Guangzhou area, the genetic diversity of *V. cholerae* O1 was studied with 276 strains isolated from cholera patients, carriers, and environments during 2001–2005 [125]. As evidenced from this study, *V. cholerae* strains were categorized into three types with pathogenicity gene profiles of *ctxA*⁺ *tcpA*⁺ *ace*⁺ *zot*⁺ (type A), *ctxA*[−] *tcpA*[−] *ace*⁺ *zot*⁺ (type B), and *ctxA*[−] *tcpA*[−] *ace*[−] *zot*[−] (type C) using a multiplex PCR. The distribution of type A profile was detected in 68.5% cases with mild symptom and 22% in carriers and type C in 64% cases with mild symptom and 36% in carriers. With the environmental strains, type C profile was common (55%) than type B (36%) [125].

The incidence and pathogenic properties of *V. cholerae* non-O1, non-O139 have extensively been reported with the indication that the proportion of their incidence is constantly increasing [126–128]. Even though the non-O1, non-O139 lacks virulence properties of O1/O139, virulence machineries such as heat-stable toxin [129] and type III secretion system [128] may have some clinical significance. Some of the non-O1, non-O139 strains isolated from River Narmada at Jabalpur, India, harbored the *ctxA* and *tcpA* genes indicating the potential reservoir of the pathogen [130]. Toxigenic non-O1, non-O139 strains harboring CTX genetic element, El Tor allele of *hlyA*, and *stx* were identified in non-cholera-endemic regions such as Kerala, India [131]. However, incidences of such strains in clinical cases are not reported. The presence of viable but non-culturable (VBNC) forms of *V. cholerae* O1 in many aquatic environs was correlated well with the incidence of cholera in Vellore, southern India [132].

After its last dominance in 1989, sporadic infections caused by *V. cholerae* O1 Inaba serotype were recorded in Kolkata during 1998–1999 [133]. Ribotyping and PFGE results showed that the Inaba strains were evolved from the Ogawa serotype prevailed that time [133]. During 2004–2005, cholera caused by the Inaba serotype was recorded in 15 states of India, mostly associated in the form of outbreaks [15]. These Inaba strains had unique PFGE (pulsotype H1) and ribotype (RIV) profiles that were not recorded before. After its first appearance in July 2004, the Inaba

serotype completely replaced the dominant Ogawa serotype from May 2005 in Kolkata [134]. These Kolkata Inaba strains belonged to a new clone in the ribotyping as well as PFGE, identical to the Delhi strains and had a CTX prophage with two RS elements. Similar results were obtained with Inaba strains isolated in Trivandrum, southern India, except for ribotyping, which showed that the Inaba and Ogawa strains were similar [135].

Cholera outbreaks were recorded during the late 1990s in several districts of Teheran, Iran [136]. Though the isolated strains belonged to Ogawa serotype and carried two or three copies of *ctx*, ribotyping results showed prevalence of single clone during the outbreaks. Most of the Iranian *V. cholerae* O1 strains (33–96%) harbored a large plasmid and displaced limited number of clones as evidenced from ribotyping and PFGE analysis [137]. *V. cholerae* O1 Inaba strains collected from several cholera outbreaks in Iran during 2005 were clonally identical as detected by PFGE and ribotyping [94]. Recently, frequency in the isolation of O139 serogroup is less globally. Few O139 strains isolated in India during 2003 shared similar PFGE profile with strains isolated in 2000 [93]. However, organization of the tandemly arranged prophages such as CTX (El), CTX (Cal), and truncated CTX (Cal) that had no *ctxAB* was unique in majority of the O139 strains.

Conventionally, the classical and El Tor biotypes of *V. cholerae* O1 are identified with phenotypic markers. Presently, the molecular markers were developed to differentiate the biotypes exploiting the nucleotide difference in the sequences of gene encoding the toxin-coregulated pilus (*tcpA*), *rstR*. The repeat in toxin gene *rtxC* is absent only in the classical biotype. Cholera outbreak in Mozambique during 2004–2005 was caused by hybrid strains between classical and El Tor biotypes, which were different from Bangladeshi classical strains [138]. The Mozambique hybrid strains had *rstR* allele specific for classical biotype. The cholera toxin (CT), encoded by *ctxA* and *ctxB* genes, is the main virulence factor in *V. cholerae*. Based on the amino acid residue substitution at positions 39, 46, and 68 in the B subunit of the CT (CtxB), three genotypes were identified [109]. The classical and US Gulf Coast El Tor strains were classified as genotype 1 and the Australian El Tor strains as genotype 2. Almost all the seventh pandemic El Tor strains belong to genotype 3. El Tor strains having classical CtxB has been confined to US Gulf Coast strains. In 2004, hybrid El Tor strain producing classical CT was first identified from cholera patients in Matlab, Bangladesh, and in Beira, Mozambique. In 2006, this hybrid strain replaced the prevailing El Tor biotype in Bangladesh [139]. Subsequently, the hybrid El Tor strains that produce the classical CT has been reported in many Asian and African countries [140, 141]. Genomic changes in *V. cholerae* possibly provide better survival in the environment and surpass the immuno-impediment of the host. However, the epidemiological significance of this hypothesis should be studied in detail.

2.9 Seroepidemiology

The vibriocidal antibody titer has been used as an epidemiological marker for the determination of recent cholera infection. Such markers are generally used in the cholera vaccine trials to confirm the efficacy of the vaccines. A Mexican study has

demonstrated the use of seroepidemiology in studying prevalence of cholera [142]. US Gulf Coast study demonstrated that the vibriocidal titers were significantly higher in Vietnamese subjects than in non-Vietnamese subjects [143]. This indicates that the focus of cholera is confined to specific geographical regions. Compared to the serum IgG, the levels of IgA against B subunit of CT, LPS, and TcpA were high in household contacts of patients infected with *V. cholerae* O1 in Bangladesh [144]. Strong immunoglobulin IgA and IgM antibody-secreting cell (ASC) responses were reported in cholera patients against the homologous serogroup [145]. Vibriocidal assay also showed the same trend indicating that these responses did not evoke cross-protection between the infections caused by O1 and O139 serogroups.

2.10 Prospects of Cholera Vaccines in Asia

During disaster periods, it was always difficult to formulate an effective disease control measure, as this needs proper planning, extended help from local government, supply and stocking of life-saving drugs/vaccines. Under this complex situation, vaccine campaign is difficult. Surpassing all the difficulties, an oral cholera vaccine was given to people affected by tsunami in Aceh Province, Indonesia, during 2004. Almost 69% of the population received the vaccine in two doses [146]. After this successful implementation of the vaccine program, the WHO has issued recommendations in 2004 for the use of oral cholera vaccine during emergencies [146]. In cholera-endemic areas, there is an increasing demand for the oral cholera vaccine at moderate prices as protection efficacy is more than 50% and herd protection studies indicated that the unvaccinated persons were also benefitted as the overall incidence of cholera was less [147, 148]. Investigations on the safety and immunogenicity of an anti-O1 and anti-O139 killed oral whole-cell cholera vaccine (biv-WC) in Vietnam showed promising results. This vaccine gave fourfold increase in vibriocidal antibody titer in 60 and 40% of the adults against O1 and O139, respectively [149]. Similarly, the vibriocidal response was high among children 1–12 years of age with 90 and 68% seroconversion against O1 and O139 serogroups. The killed whole-cell oral cholera vaccine study conducted during 1998 in Hue, Vietnam, revealed its long-term protection (3–5 years) with high efficacy (50% range 9–63%) [150].

An analysis of cholera vaccine trials with rCTB-WC showed protection in 61–86% of people living in endemic areas for 4–6 months and up to 3 years at low levels [151]. This vaccine was proposed for workers employed in the relief or refugee camps or for those who will be traveling in epidemic areas and for those who have no medical care option. An effective vaccine against cholera has been used for public health purposes in Vietnam since the 1990s. In a cholera-endemic area in Kolkata, India, safety and immunogenicity of the reformulated bivalent killed whole-cell oral vaccine with *V. cholerae* O1 and O139 was tested in a double-blind, randomized, placebo controlled trial (healthy adults). Following immunization, 53% of adult and 80% of children vaccines showed a ≥ 4 -fold rise in serum *V. cholerae* O1 vibriocidal antibody titers [152]. However, the vibriocidal antibody titers of post-immunization

among vaccinees were less for *V. cholerae* O139. Remarkably, the adverse reaction for this vaccine was similar among vaccine and placebo recipients in both age groups. One of the recent studies on live cholera vaccine (VA1.3) showed that the seroconversion rate was 57% with high anti-CT response (77%) and the vaccine was highly safe in the adult volunteers [153]. A modified killed-whole-cell-oral cholera vaccine trial (with a mixture of formalin-killed and heat-killed *V. cholerae* O1 [classical and El Tor biotypes] and O139 strains) in Kolkata, India showed 68% protection efficacy among all age groups [153a]. This vaccine is licensed in India as Shancal® (Shantha Biotechnics, Hyderabad, India).

2.11 Traditional Medicine and Food Habits for Prevention of Cholera

During the fifth decade of the nineteenth century, Dr. J. Collins Browne, an army surgeon worked in British India, invented a patented medicine Chlorodyne, a compound of tincture of chloroform and morphine for the treatment of cholera patients [154]. Chlorodyne was imported to Japan during 1870 and 1873. Dr. Jyun Matsumoto supported the preparation of traditional medicine and a local preparation named “Shinyaku” that resembles Chlorodyne was marketed in Japan in 1872. In the Japanese unknown formulation, morphine hydrochloride, diluted hydrocyanic acid, and tincture of Indian hemp were replaced [154]. However, this formulation was not popular in many countries where epidemics of cholera were frequent.

Traditional herbal medicines (Kampo formulations) are being used in China and Japan for many centuries. The gallate compound from *Rhei rhizoma* inhibited cholera toxin effect in the tissue culture and fluid accumulation in the rabbit ileal loop assay [155]. The synthetic gallate has shown toxin in vivo and in vitro inhibitory effects and thus the Kampo formulation seems to be an effective adjunctive therapy with oral rehydration solution [155]. Soy sauce (Shoyu) was found to have antimicrobial activity against *V. cholerae* and other enteric bacteria along with other beneficial properties such as antihypertensive, anticarcinogenic, anticataract, antioxidant, and antiallergen [156]. Many useful herbal formulations for the treatment of cholera and other diarrheal diseases have been lost during passage of time and generations of people. Unfortunately, there was no recording system in primeval days and such formulations were kept secret for the benefit of professionals those practiced herbal medicine.

2.12 Control Measures and Health-Care Systems

For prevention of infectious diseases including cholera, the Chinese health-care model is being considered in developing countries. During 1940s, the mortality rate in China was 20 and 30–40% in children less than 1 and 5 years of age, respectively. The health care was given prioritized during later years and strengthened in 1965 after Cultural Revolution, which resulted in increasing the life span from

40 to 70 years and reducing the infant mortality very less [157]. The main health-care objectives considered in the Chinese system include the following: improving access to health care in rural areas, prevention of infection, emphasis on traditional medicine, and utilizing manpower rather than technical power in the health-care system [157]. The same system was proposed for African countries [158]. In Indonesia, several control measures were made in the anticipation of outbreaks in refugee camps. As a result of such activities, no cholera or typhoid cases were detected through routine surveillance [159].

In cholera-endemic regions, the case-control studies conducted to determine the etiological agent of outbreaks often lead to multiple sources or other sources such as river waters were periodically found to be one of the vehicles [160]. Asymptomatic cholera carriers might play an important role in the passive spreading of the disease. In Hong Kong, early investigations were made for the detection and treatment of carriers to prevent spread of sporadic cholera [161]. Food handlers were responsible for large-scale cholera outbreaks in Thailand [162]. The impact of cholera and other enteric diseases was assessed by providing deep-well tap water (DWTW) through household taps in Qidong village in China [163]. In this study, the overall incidence of enteric infection was 39% lower than the control region that had surface water supply. However, the initial cost of establishing DWTW is prohibitive due to many reasons. Cholera outbreaks in Tumpat and Kelantan, Malaysia, during 1990 were related to the use of Kelantan river water and consumption of river clams [164]. Several carriers were also identified in this outbreak investigation.

In Bangladesh, use of tube-well water played a significant role in reducing the cholera mortality in rural areas [67]. The number of cholera cases in rural areas of Bangladesh was significantly reduced when a simple filtration using old sari was tested that effectively removed the plankton and other particulate matter from the natural waters [165, 166]. Despite the rural health improvement scheme, cholera was endemic in Malaysia in 2002 as most of the rural population relied on river water supply [167]. This suggests that sanitation interventions were not as effective in reducing the water-borne diseases such as cholera. Successive O139- and O1-mediated cholera appeared in Pakistan during 2002–2003 were mainly due to person-to-person contact and through contaminated water reservoirs [10]. As the cholera management was given priority in the Vietnam Health sector, the annual mortality rate reduced from 2.0–9.65% during 1979–1983 to 1.8% in the later periods [168]. An outbreak of cholera associated with a tribal funeral in Irian Jaya, Indonesia, has been reported [169]. Generally, reports of cholera associated with attending funeral are very less in Asian regions as the burial practice of the death victims and rituals are different from African region. The case-control studies for cholera risk factors were found to be similar for O1- and O139-mediated cholera that include consumption of untreated water, uncooked pork or seafood, and food served in large gatherings [162, 170, 171]. Among children less than 6 months of age, protection associated with breast-feeding has been demonstrated for cholera and the mechanisms are related to risk of supplementary feeding that may introduce pathogens and/or immunoprotection offered by the breast milk through immunoglobulins [172].

2.13 Conclusion

Over the years, the incidence of cholera has been reduced in many Asian countries and the mortality rate has also gone down. The role of phenotypic and genetic changes in *V. cholerae* O1 and O139 serogroups and their increased incidence in few countries have not been studied in detail. Antimicrobial resistance in *V. cholerae* is emerging in Asia and this can be controlled by judicious use of drugs in the management of cholera. Several efficient methods in managing cholera outbreaks have been implemented in rural and urban areas after rigorous efficacy studies including introduction of new oral vaccines, supply of safe drinking water, sanitation, and timely administration of ORS. Success of these interventions depends on maintenance and regular practice at the community level.

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Epidemiological and Molecular Aspects on Cholera

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2011, XII, 372 p., Hardcover

ISBN: 978-1-60327-264-3