
Michael D. Lewis,1 Oralak Serichantalergs,1 Chittima Pitarangsi,1 Niphon Chuanak,1 Carl J. Mason,1 Laxmi R. Regmi,2 Prativa Pandey,2 Ranjan Laskar,3 Chandrika D. Shrestha,1 and Sarala Malla4

1Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand; 2Bharatpur Zonal Hospital, 3CIWEC Travel Clinic, 4Bharatpur College of Medical Sciences, and 5Nepal National Public Health Laboratory, Kathmandu, Nepal

Background. In the summer of 2002, a total of 5963 cases of typhoid fever were recorded in Bharatpur, Nepal (population, 92,214) during a 7-week period. A team from the Armed Forces Research Institute of Medical Sciences in Bangkok, Thailand, and the CIWEC Travel Medicine Clinic (Kathmandu, Nepal) assisted the Nepal National Public Health Laboratory (Kathmandu, Nepal) in the further investigation of this large, explosive febrile disease outbreak.

Methods. Investigators conducted a thorough epidemiologic and laboratory investigation to assess the size and scope of the outbreak. In addition to subculturing of previously collected samples, blood samples were obtained from 100 febrile patients, and culture and susceptibility testing were done by standard laboratory methods. Pulsed field gel electrophoresis (PFGE) and plasmid analysis were done.

Results. The majority of the isolates, including 1 from the municipal water supply, were multidrug resistant. The minimum inhibitory concentrations (MICs) of ciprofloxacin ranged from 0.19 μg/mL to 0.125 μg/mL. With use of PFGE, all isolates, including isolates from the water supply, showed an analytical similarity of 96%–100%. Multidrug-resistant isolates had a plasmid encoding for resistance, and those with resistance to nalidixic acid had a single-point mutation.

Conclusions. To the best of our knowledge, this outbreak is the largest single–point source outbreak of multidrug-resistant typhoid fever yet reported, and it was molecularly traced to the city’s single municipal water supply. Isolates were uniformly resistant to nalidixic acid, there was a decrease in their susceptibility as measured by MIC of fluoroquinolones, and 90% of isolates obtained were resistant to >1 antibiotic.

Salmonella enterica serotype Typhi continues to be a major public health problem in much of the developing world, especially in Asia, and the global situation has steadily worsened since the emergence of multidrug-resistant strains in 1989 [1, 2]. For many years after 1948, chloramphenicol was the first-line drug of choice for the treatment of typhoid fever. After chloramphenicol-resistant outbreaks occurred in Mexico and India in the 1970s, an alternative treatment for typhoid fever was a combination of ampicillin and trimethoprim.

Although typhoid fever is endemic in most developing Asian countries, outbreaks of drug-resistant typhoid fever have been recorded in India, Pakistan, Bangladesh, Vietnam, and Tajikistan [1–4]. Of particular concern is the development of resistance to quinolones and decreased susceptibility to fluoroquinolones. A large scale, multiyear, multifocused outbreak of multidrug-resistant typhoid fever was reported from Tajikistan during 1996–1998 [3–4].

Nepal is a poor South Asian country that is located between India and China. Municipal services, especially outside of the capital Kathmandu, have been deteriorating as the Maoist insurgency has gained momentum in recent years. In late May 2002, the Early Warning Reporting System (EWARS), established in Nepal with support from the United States Agency for International Development, noted a dramatic increase in the number of febrile patients in the 92,214-person city of Bharatpur, the gateway into the Chitwan National Preserve, located 50 miles southwest of Kathmandu.
EWARS was established to assist the government in recognizing and controlling outbreaks of acute vector-borne diseases and has the flexibility to allow reporting of unusual events. On 24 May 2002, the local hospital medical records officer began reporting a large increase in the number of febrile patients.

Initial reports from the Bharatpur Zonal Hospital (BZH) were that all blood cultures showed no growth, and the Bharatpur College of Medical Sciences Hospital (CMSH) reported 2 deaths of student nurses from smear-negative malaria. An immediate investigation by local government health officials concluded that there was no typhoid fever or malaria among the 4000 patients seen in the first 10 days of June. The Nepalese Ministry of Health’s National Public Health Laboratory (NPHL) then sent a team to investigate. Six blood cultures obtained at the BZH that showed no growth were randomly selected from the BZH laboratory and were taken to Kathmandu for incubation. Growth of S. enterica serotype Typhi occurred in all 6 cultures after incubation at the NPHL. They were initially reported to be resistant to all antibiotics, including ciprofloxacin, according to the disk diffusion method, although no zone diameters were recorded. Additionally, the NPHL obtained 10 samples from various locations in the Bharatpur municipal water system, 2 of which yielded S. enterica serotype Typhi on culture. Because of the positive findings, Ministry of Health officials recommended on 11 July that the Bharatpur authorities check the single water distribution plant for adequate chlorination. Bharatpur officials noted that basic chlorination had been neglected for an indeterminate period. The outbreak continuing, >5000 patients affected, and no definitive cause established, at the request of His Majesty’s Government, a team of medical researchers from the Armed Forces Research Institute of Medical Sciences (AFRIMS) in Bangkok, Thailand, and the CIWEC Travel Medicine Clinic (Kathmandu) assisted the NPHL in the further investigation of this large outbreak in the Bharatpur area.

METHODS

Epidemiological assessment. Daily numbers of patients at the BZH and the CMSH emergency and outpatient departments were recorded by the medical records officer for the period 24 May through 13 July 2002. On 25–26 June and again in November 2002, the AFRIMS team assessed the size and scope of the outbreak by reviewing EWARS reports, hospital records, and emergency department log registers dating from 1 May 2002. The case definition used was fever known or clinically suspected to be caused by S. enterica serotype Typhi as recorded by the admitting physician. Data available from emergency department records and hospital registers at the BZH and the CMSH included date of presentation to the hospital, age, sex, city ward of residence, and known or suspected diagnosis. Demographic data were not available from the hospital outpatient departments. Population data down to the level of wards for the city of Bharatpur and a map of the city showing ward boundaries (figure 1) were obtained from city authorities. Because of the limited time available to conduct an investigation and determine the cause of the outbreak, a case-control study was not possible.

Laboratory investigation. During the AFRIMS team onsite investigation, the BZH laboratory technicians obtained blood samples from the first 100 patients who presented to the hospital with a body temperature of ≥38°C (≥100°F). To test for malaria, 2 thick and thin blood film slides were made and read by an AFRIMS expert microscopist. The remaining blood (often only 0.5–2.0 mL after physician-ordered tests had been performed) was placed in a single Bactec culture bottle (BacT/Alert SA; Organon Teknika) and incubated at 37°C.

Routine plating, isolation, and identification were done, and confirmation of salmonella was performed using serological testing with Salmonella antisera (S&A Reagents Lab). Antimicrobial susceptibility testing (BBL Sensi-Disc; Becton Dickinson) was performed using NCCLS standard disk diffusion methods [5]. Fifteen antimicrobial agents were evaluated: azithromycin, nalidixic acid, ciprofloxacin, ampicillin, chloramphenicol, colistin, gentamicin, kanamycin, neomycin, streptomycin, sulfizoxadole, tetracycline, amikacin, trimethoprim/sulfamethoxazole, and ceftriaxone. MIC tests (Etest strips; AB Biodisk) for ofloxacin, ciprofloxacin, and ceftriaxone were completed for all isolates.

Samples for subcultures were also obtained from 25 cultures with growths suspected to be S. enterica serotype Typhi at the Bharatpur CMSH. At the NPHL in Kathmandu, subcultures were performed for the 6 isolates previously obtained from the Bharatpur area and the 2 isolates from Bharatpur municipal water supply. A separate group of 61 NPHL isolates were subcultured for use at AFRIMS as background information, representing typhoid fever–related isolates recovered throughout Nepal during the previous 2 years.

PFGE was conducted at AFRIMS by means of the CHEF-DRIII system (Bio-Rad), as described by the standard PulseNet protocol for Salmonella species from the Centers for Disease Control and Prevention (Atlanta, GA). The band difference was interpreted using the method of Tenover et al. [6]. The similarities of the restriction fragment–length polymorphism were analyzed using BioNumerics software, version 2.5 (Applied Maths) to produce a dendogram. The similarity between 2 restriction fragment–length polymorphisms was determined with a Dice coefficient.

Plasmid analysis was performed using a modification of the method described by Birnboim and others [7–9]. DNA sequence analysis of DNA gyrase quinolone resistance–deter-
Figure 1. City map of Bharatpur, Nepal, obtained from city officials, showing locations of the 14 city wards, the sole water treatment and distribution plant, and the Narayani river.
Mining regions was conducted in accordance with methods described by Hirose et al. [10, 11] to determine if mutations existed in the gyrA (codon 83) and gyrB (codon 464) genes.

**Municipal water supply assessment.** In addition to the samples obtained by the NPHL earlier during the outbreak (before subsequent chlorination at the water distribution plant), water samples were obtained by the AFRIMS team on 26 June from each of the 14 wards in the city of Bharatpur and were tested for the presence of residual chlorine using a colorimetry method in accordance with manufacturer’s instructions (Chlorine Colorimeter Model 1200-UDV-CL; LaMotte). Samples with chlorine levels of <0.30 mg/L were cultured on MacConkey and Hektoen Enteric agar, as outlined above.

**RESULTS**

**Epidemiological assessment.** The zonal hospital medical records officer recorded a total of 5963 febrile cases confirmed or clinically suspected to be cases of typhoid fever at the 2 Bharatpur area hospitals, resulting in a crude attack rate of 64.9 cases per 1000 population during a 7-week period (24 May–10 July) (figure 2). These data include 1528 emergency department and 1928 outpatient department patients at the BZH and 656 emergency department and 1851 outpatient department patients at the CMSH. The peak number of patients was noted on 9 June (273 patients). The largest burden fell on Bharatpur ward 10, the most populous ward in the center of the city and also the location of the isolate obtained from the municipal water supply. Data for a total of 2135 patients from ward 10 were recorded, and a crude attack rate of 122.7 cases per 1000 population was calculated. The wards with the lowest number of patients were ward 14 (a total of 31 patients; attack rate, 10.4 cases per 1000 population) and ward 2 (a total of 90 patients; attack rate, 9.6 cases per 1000 population). The overall attack rate for the 14 wards of Bharatpur was 48.9 cases per 1000 population. Only 4 deaths were recorded, 2 at the BZH (a 9-year-old girl and a 6-year-old boy) and 2 at the CMSH (two 24-year-old women), and only 3 patients required surgery for complications caused by typhoid fever.

It is estimated that 60%–90% of patients with typhoid fever do not receive medical care or are treated as outpatients [2]. According to data presented in several past studies [12–14], it is estimated that, in cases of typhoid fever, blood cultures have positive results only ∼40% of the time. During the period for which AFRIMS investigators were processing blood cultures, 35% of the febrile patients had positive culture results, probably because of the small amount of blood available for culture. An estimation of the actual size of the outbreak, therefore, ranges from as few as 2087 (35%) of the 5963 patients with typhoid fever to as many as 59,830 persons (assuming that only 10% of individuals with typhoid fever sought medical care) in a city of 92,214 (i.e., 65% of the general population) during this 7-week period. Our epidemiological estimate on the basis of EWARS reports, hospital records, and emergency department

![Figure 2](https://academic.oup.com/cid/article-abstract/40/4/554/353277)
log registers is a more likely measure of the disease burden, given the circumstances and assumptions.

**Laboratory.** An assessment of the laboratory at the BZH revealed that the incubators being used for blood cultures were of insufficient temperature and were probably the reason for false-negative culture results locally. Malaria smears were obtained from 97 of the 100 febrile outpatients seen by AFRIMS investigators at the BZH laboratory on 26–27 June over a 6-h period, and no malaria parasites were found. A total of 98 blood cultures were obtained after samples were processed for physician-ordered laboratory testing. Even with the small amount of blood remaining for culture (0.5–2.0 mL), 33 cultures grew *S. enterica* serotype Typhi, and 1 culture grew *S. enterica* serotype Paratyphi. It is recommended that 5–10 mL of blood should be used for 1–2 culture bottles [15, 16]. According to previous studies, it is estimated that blood cultures have positive results for only 40% of persons with typhoid fever [12–14] and that there is a 29% decrease in positive cultures per mL as less blood is cultured [17]. It is possible that as many as 85 (88%) of 97 febrile patients seen during a 6-h period on 25–26 June actually had typhoid fever.

All 6 subcultured outbreak isolates from the NPHL were confirmed as *S. enterica* serotype Typhi. Of the 2 water samples obtained from Bharatpur by the NPHL early in the outbreak, 1 sample (obtained from ward 10) was confirmed as *S. enterica* serotype Typhi, and the other was no longer viable. Of the 25 isolates suspected to be *S. enterica* serotype Typhi obtained from the CMSH, 21 were confirmed at AFRIMS, and 1 was identified as *S. enterica* serotype Paratyphi. The other 3 isolates obtained from the CMSH were identified as nonenteric pathogens.

Of the 61 NPHL isolates obtained throughout the country and representing background information not associated with the outbreak, 25 were identified as *S. enterica* serotype Typhi, and 3 were identified as other *Salmonella* species. The rest were no longer viable or were nonenteric pathogens.

All *S. enterica* serotype Typhi isolates were Vi-phage type E1, the most common multidrug-resistant phage type found in South Asia [18]. The most common antimicrobial-resistance pattern of the isolates was resistance to ampicillin, chloramphenicol, streptomycin, sulfazoxidole, tetracycline, trimethoprim/sulfamethoxazole, and nalidixic acid (R-type ACSSu-TSXTN). This pattern accounted for 28 of the 33 isolates obtained by AFRIMS, whereas the other 5 isolates had different patterns; however, all isolates were resistant to nalidixic acid (table 1). Of the 33 patients, 12 reported having taken an antibiotic (8 took ciprofloxacin or ofloxacin). All 6 subcultured outbreak isolates and the 1 municipal water supply isolate obtained by the NPHL had the ACSSuTSXTN resistance pattern, as did 17 of the 21 CMSH isolates (the other 4 were resistant to nalidixic acid).

According to disk diffusion testing, all of the isolates appeared to be susceptible to 5 μg/mL ciprofloxacin (zone diameters, 22–24 mm), although there were reports of clinical failure of treatment with ciprofloxacin and ofloxacin. With use of the Etest strip method, MICs of ciprofloxacin were measured at 0.19 μg/mL for 53 isolates and 0.125 μg/mL for 8 isolates; MICs of ofloxacin were measured at 0.38 μg/mL for 36 isolates and at 0.50 μg/mL for 25 isolates; and MICs of ceftriaxone were measured at 0.094 μg/mL for all 61 isolates.

All 33 isolates obtained by AFRIMS, all 6 isolates obtained from Bharatpur by the NPHL, all 21 isolates obtained from the CMSH, and the water sample obtained by the NPHL from the municipal water supply showed a similarity of 96%–100% according to PFGE analysis (figure 3). The Xhol-digested and SpeI-digested DNA of *S. enterica* serotype Typhi from the outbreak strains produced 15–24 fragments with lengths of 48.5–533.5 kb. Plasmid analysis showed that all multidrug-resistant isolates contained an ~150-kb plasmid. This plasmid was not present in strains that showed only nalidixic resistant. All isolates obtained during the outbreak were uniformly found to have a single-point mutation in the *gyrA* gene (83), from TCC to TAC (Ser–Tyr), and no mutations in the *gyrB* gene (GenBank accession number, AY584860). Other samples from the NPHL collected before the outbreak showed a wide genetic variation according to PFGE and resistance pattern analysis (figure 3).

**Municipal water supply assessment.** As noted, 1 water sample obtained by the NPHL from ward 10 early in the outbreak was confirmed as *S. enterica* serotype Typhi at AFRIMS (R-type ACSSuTSXTN). The municipal water supply in Bharatpur is intermittent and is turned on twice per day for 1 h. Municipal water supply pipes are run in the open sewer system throughout the city. Water is often stored in local cisterns for later use, and multiple instances were noted in which taps were left open and water was left running into the streets. Of the 14 water samples obtained by AFRIMS on 26 June (after the water

<table>
<thead>
<tr>
<th>Source of isolate</th>
<th>Amp-Chl–Stm–Sul–Tet–Ax</th>
<th>NA</th>
<th>Chlor–Tet–Ax</th>
<th>Tet–NA</th>
</tr>
</thead>
<tbody>
<tr>
<td>BZH/AFRIMS</td>
<td>33</td>
<td>28</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>CMSH</td>
<td>21</td>
<td>17</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>BZH/NPHL</td>
<td>6</td>
<td>6</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Water sample</td>
<td>1</td>
<td>1</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Total</td>
<td>61</td>
<td>52</td>
<td>6</td>
<td>2</td>
</tr>
</tbody>
</table>

**Table 1.** Distribution of outbreak-related *Salmonella enterica* serotype Typhi isolate resistance patterns according to source of isolate.

**NOTE.** Amp, resistance to ampicillin; AFRIMS, Armed Forces Research Institute of Medical Sciences; BZH, Bharatpur Zonal Hospital; Chl, resistance to chloramphenicol; CMSH, Bharatpur College of Medical Sciences Hospital; NA, resistance to nalidixic acid; SMZ-TMP, resistance to trimethoprim/sulfamethoxazole; Stm, resistance to streptomycin; Sul, resistance to sulfazoxidole; Tet, resistance to tetracycline.
Figure 3. Relatedness of PFGE patterns generated by XbaI cleavage, according to analysis with Bionumerics software, version 2.5 (Applied Maths). The similarity between 2 restriction fragment–length polymorphisms was determined with a Dice coefficient. Isolates H–Q and isolate S were obtained from Bharatpur, Nepal, during the typhoid fever outbreak and show a 96%–100% similarity. Isolate J was obtained from the municipal water supply. Isolate R was found to be circulating in Nepal before the Bharatpur outbreak. All other isolates represent background typhoid fever–related isolates obtained from a collection of isolates cataloged at the Nepal Public Health Laboratory, Kathmandu. Amp, resistance to ampicillin; Chl, resistance to chloramphenicol; Gm, gentamicin; NA, resistance to nalidixic acid; SMZ-TMP, resistance to trimethoprim/sulfamethoxazole; Tet, resistance to tetracycline.

system had been chlorinated), residual chlorine levels ranged from 0–0.61 mg/L. The chlorine levels for 12 of 14 samples were <0.30 mg/L, and for 8 samples, they were <0.10 mg/L. No additional isolates of S. enterica serotype Typhi were cultured from these samples.

CONCLUSIONS

We believe this multidrug-resistant typhoid fever outbreak in Bharatpur is the largest single-point source, multidrug-resistant typhoid fever outbreak reported in the literature. Although the actual number of patients with typhoid fever will never been known, 5963 patients with confirmed or clinically-suspected typhoid fever were seen at the 2 city hospitals during a 7-week period (24 May–13 July 2002), giving a crude attack rate for the city of 64.9 cases per 1000 population. The outbreak was traced with molecular epidemiological methods to a single source—the sole municipal water supply. In addition to isolates from Bharatpur being uniformly resistant to nalidixic acid (a first-generation quinolone), there was a decrease in susceptibility as measured by the MICs of ciprofloxacin and ofloxacin (both of which are fluoroquinolones) and the MIC of ceftriaxone; 90% of the isolates obtained were resistant to >1 antibiotic.

Because of the widespread existence of multidrug-resistant typhoid fever, ciprofloxacin has become the recommended che-
motherapy for patients with typhoid fever. However, concerns have developed in the past decade about the continued clinical effectiveness of fluoroquinolones in the presence of nalidixic acid resistance [2, 19–21]. Local physicians in Bharatpur reported clinical failures of fluoroquinolone therapy when used alone and were using intravenous ofloxacin plus ceftriaxone for treatment. This probably led to the decreased mortality seen during the outbreak, although it apparently had no bearing on the morbidity [19]. Although according to NCCLS standards, the isolates were not resistant to fluoroquinolones, these standards have recently been called into question [20, 21]. Aaerstrup et al. [21] suggest that a breakpoint of 0.125 mg/L for fluoroquinolones should be used when evaluating the MIC for salmonellae. Using that suggested standard, this outbreak would also be classified as multidrug resistant and fluoroquinolone resistant.

With use of background isolates from the NPHL, we are able to show that multiple strains of S. enterica serotype Typhi were circulating in Nepal before the outbreak. The mechanism for tetracycline resistance is the acquisition of a plasmid encoding resistance, whereas quinolone resistance is conferred by a mutation in the genes encoding DNA gyrase (gyrA or gyrB) [2, 6–11]. Several strains were found to be circulating in Nepal that had one type of resistance or the other but not both. All isolates obtained from Bharatpur at the time of the outbreak possessed both plasmid-encoded resistance and a single-point gyrA mutation. This outbreak involved the first known strains isolated in Nepal with both resistance mechanisms.

Through PFGE analysis, we were able to trace the outbreak to the single municipal water supply. An intermittent supply of water, such as existed in Bharatpur, allows for the cross-contamination of sewage and water-distribution systems, leading to serious public health consequences [22]. It is unlikely that the water was contaminated at the distribution plant, but the failure to adequately chlorinate the distributed water, together with cross-contamination, had devastating effects. Following the recommendation by the NPHL to ensure adequate chlorination at the water distribution plant, no additional typhoid fever–related isolates were recovered from the municipal water system when it was rechecked by AFRIMS, although the amount of chlorine residual at the tap was often negligible.

The ongoing civil unrest in Nepal continues to exact a toll on its citizens. This outbreak represents a breakdown of the basic public health and civil engineering infrastructure and is a poignant reminder of the consequences that occur when basic community infrastructure is neglected. Although typhoid fever is endemic in Nepal, a combination of inadequate chlorination of the only municipal water source and its intermittent supply was directly responsible for the massive scope of this outbreak. Not only is the Bharatpur outbreak the largest single-point source outbreak reported in the literature, but it possibly represents a warning concerning the future of typhoid fever. Multidrug-resistant typhoid fever is not only becoming more prevalent in Asia, but the susceptibility to the current first-line treatment, fluoroquinolones, is decreasing and will soon need to be reevaluated. It appears that ciprofloxacin may soon join chloramphenical and ampicillin as a failed treatment option in the war against typhoid fever.

Acknowledgments

We thank Mr. Ramesh Prashad Adhikari (Senior Public Health Officer; Bharatpur, Nepal), Dr. B. N. Chaudhary (Bharatpur Zonal Hospital; Bharatpur, Nepal), and Dr. A. C. Pataswary (Bharatpur College of Medical Sciences; Bharatpur, Nepal); without their assistance, the field assessment of this outbreak would not have been possible. In addition, Dr. R. Scott Miller (Armed Forces Research Institute of Medical Sciences; Bangkok, Thailand) reviewed this manuscript and provided valuable editorial feedback.


Potential conflicts of interest. All authors: no conflicts.

References


