

## Detection of *Legionella* Species from Rainwater on Roads in Bhutan

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We collected samples of rainwater on road in Bhutan and determine whether or not *Legionella* species is present by colorimetric PALSAR method. Results showed all six samples were positive for *Legionella*. To our knowledge, this is the first report which shows the presence of *Legionella* species in Bhutan. *Legionella* species is a major cause of severe community-acquired pneumonia and can be estimated to be widely scattered in all over the world. However, reports are strongly biased from specific countries including European countries, the United States, and Japan. We hope to help physicians to choose proper antibiotics especially for patients with severe pneumonia considering about the possibility of legionellosis by showing evidences that *Legionella* species can be detected from diverse corners of the world, which may lead to reduction of preventable deaths.

**Key words:** *Legionella* species, the Kingdom of Bhutan, rainwater on roads, colorimetric PALSAR method

### 1. Introduction

*Legionella* has been detected from potable water, cooling towers, water taps and showers, humidifiers, whirlpool baths, spas, medication nebulizers, dental units, evaporator compartments of an air-conditioning system, fountains, potting soil, streams, ponds, lakes, springs, ground water, and oceans.<sup>1-20)</sup> It was also detected in water at high altitudes of over 4000 meters above sea level and even in Antarctic lakes.<sup>21,22)</sup> Factors known to enhance colonization of *Legionella* include humidity, warm temperatures (25–42° C), pH 5.5-9.2, 0.3-9.6 ppm of dissolved oxygen in water, low flow, scale, and sediment.<sup>2,23-30)</sup> Inhalation of aerosols

containing *Legionella* or microaspiration of water contaminated with the pathogen can lead to legionellosis, a collection of infections caused by *Legionella* species. *Legionella* accounts for 0.5-16% of community-acquired pneumonia (CAP) requiring hospitalization and 2-30% of severe CAP.<sup>31-37)</sup> Cases have been associated with potable water, cooling towers, water taps and showers, humidifiers, whirlpool baths, spas, medication nebulizers, and potting soil.<sup>38-44)</sup>

In this study, we tried to detect *Legionella* species from rainwater on roads because several reports have shown significant relationships between legionellosis and precipitation.<sup>45-47)</sup> Fisman et al. found that the



Figure 1 A moving car on a road in rainy days in Bhutan

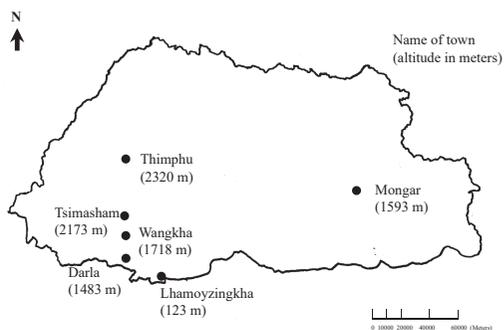


Figure 3 Map of sampling locations



Figure 2 Puddles on a road in Bhutan

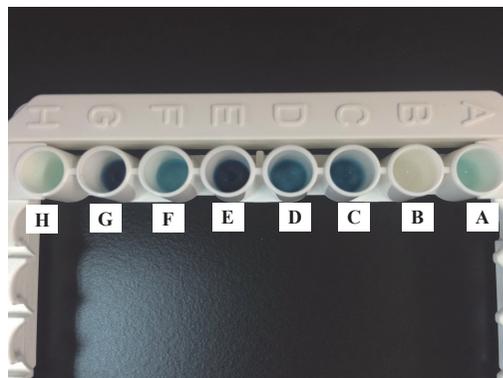


Figure 4 Detection of *Legionella* species by colorimetric PALSAR method  
Lane A, Positive Control; Lane B, Negative Control; Lane C, Sample 1 (Lhamoyzingkha); Lane D, Sample 2 (Darla); Lane E, Sample 3 (Wangkha); Lane F, Sample 4 (Tsimasham); Lane G, Sample 5 (Thimphu); Lane H, Sample 6 (Mongar).

incidence rate ratio for legionellosis 6–10 days after rainfall was 2.48 (95% confidence interval [CI], 1.30–3.12), and increased humidity (OR per 1% increase in relative humidity 1.08, 95% CI 1.05–1.11) 6–10 days prior to the identification of infected patients.<sup>45)</sup> Consistent with this, Hicks et al. reported that a 1-cm increase in rainfall was associated with a 2.6% rise (risk ratio 1.026, 95% CI 1.012–1.040) in the incidence of legionellosis.<sup>46)</sup> Moreover, Garcia-Vidal et al. reported that patients admitted to hospitals when the prior weighted median rainfall was higher than 0.42 were more likely to have *Legionella* pneumonia (OR 1.35; 95% CI 1.02–1.78;  $p=0.03$ ).<sup>47)</sup> Several outbreaks of legionellosis after heavy rainstorms or floods have also been reported.<sup>48,49)</sup> We previously reported that

*Legionella* is abundant in rain puddles on roads, particularly during warm weather.<sup>50)</sup> Indeed, a close genetic relationship has been found between *Legionella* isolates from patient sputum specimens and samples from puddles by sequence-based typing.<sup>51)</sup> Because airborne *Legionella* can survive longer at high relative humidity, after rain the organism can be sprayed into the air and increase both on the ground and in the air.<sup>50)</sup>

## 2. Methods

We collected six samples of puddles on asphalt road in Bhutan. Bhutan is located on the south face of the Himalayan range and the large portion of the country, is influenced by monsoons. The monsoon brings moist air from the Indian Ocean northward and the mountain ranges become anchors for rainfall. The rainy season usually lasts from late-June through late September. We collected the sample from the end of August through September 2016. After rainfall, water samples were suctioned from road surfaces into sterile syringes and then stored in sterile bottles (Fig. 1, Fig. 2). The collection sites were Lhamoyzinkha at 123 meters above sea level (MASL) (Sample 1), Darla at 1483 MASL (Sample 2), Wangkha at 1718 MASL (Sample 3), Tsimasham at 2173 MASL (Sample 4), Thimphu at 2320 MASL (Sample 5), and Mongar 1593 MASL (Sample 6) (Fig. 3). For reference, Ugyen Dorji, et al. reported meteorological information from 73 station sites in Bhutan. There was no station in neighboring area of Lhamoyzinkha on that report.<sup>52)</sup> Tala adjacent to Darla was reported to have mean annual temperature of 15.7° C and average annual precipitation of 3256 mm. Chukha adjacent to Wangkha and Tsimasham was reported to have mean annual temperature of 15.5° C and average annual precipitation of 1543 mm. Thimphu was reported to have mean annual temperature of 14.0° C and average annual precipitation of 671 mm. Mongar was reported to have mean annual temperature of 17.9° C and average annual precipitation of 891 mm.

In this study we used a rapid detection kit for *Legionella* species (FASMAC Co., Ltd., Kanagawa, Japan). The kit is based on coloring Probe Alternation Link Self-Assembly Reaction (PALSAR) method, targeting 16S rRNA of *Legionella* species.<sup>53,54)</sup> First, full amount of samples (Sample 1-4, 200 mL; Sample 5, 100 mL; Sample 6, 50 mL) were filtered by 0.22 µm filter paper (Merck Millipore Co., Ltd., Darmstadt, Germany). The filter paper was collected in a tube mixed with 100 µL of denaturant and vortexed for a minute. Then, the bacterial lysate was left to stand for 15 minutes inside a constant temperature bath with 37° C. After that, 10 µL of neutralizing solution was added and vortexed for two minutes to make a

conditioned bacterial lysate. Second, as a prehybridization step, 150 µL of buffer solution was added into each well of a well strip plate where a capture probe was immobilized, and the well strip plate was left to stand inside the constant temperature bath with 37° C for 30 minutes. Then, the solution was removed by tapping the plate against paper towels. Third, 40 µL of 1<sup>st</sup> hybridization reaction solution containing an assist probe was added into the conditioned bacterial lysates, a positive control, and a negative control and vortexed for two seconds. These solutions were added into the wells and left to stand inside the constant temperature bath with 37° C for 90 minutes. Then, these solutions were removed from the wells. After that, 300 µL of wash solutions were added into the wells, tapped for a minute, and removed the solution. This step was repeated six times. Fourth, 150 µL of 2<sup>nd</sup> hybridization reaction solution containing two honeycomb probes labeled with digoxigenin was added into each well and left to stand inside the constant temperature bath with 37° C for 30 minutes. Then, the washing procedure was repeated six times. Fifth, 150 µL of conjugate solution containing peroxidase-conjugated anti-digoxigenin antibody was added into each well and left to stand inside the constant temperature bath with 37° C for 60 minutes. Then, the washing procedure was repeated six times. Sixth, 75 µL of color-forming solution containing tetramethylbenzidine was added into each well and left to stand at room temperature in a place away from light for fifteen minutes. Finally, color of the sample was checked by visual judgement. Optical density was quantified using a microplate reader, Ultramark Model 550 (Bio-Rad Laboratories, California, U.S.A.) equipped with a 655 nm filter was used for absorbance measurement.

## 3. Results

Every sample except for the negative control was positive (Fig. 4). The optical density values of the samples were as follows: Positive Control 0.093, Negative Control 0.025, Sample 1 (Lhamoyzinkha) 1.454, Sample 2 (Darla) 1.147, Sample 3 (Wangkha) > 3.500, Sample 4 (Tsimasham) 0.593, Sample 5

(Thimphu) 3.269, and Sample 6 (Mongar) 0.069.

#### 4. Discussion

The results of study showed all six samples of rainwater on road in Bhutan were positive for *Legionella* species by colorimetric PALSAR method. As far as we know, this is the first report which indicates the presence of *Legionella* species in Bhutan. About the studies of legionellosis, particularly concerning is that information on the disease is strongly biased to regions where the specific testing for legionellosis is available. Although *Legionella* seems to be detected throughout the world, population-based surveillance on legionellosis is rare and the prevalence is unknown in most parts of the world. According to a population-based surveillance conducted in Ohio, the annual number of cases of legionellosis requiring hospitalization was estimated at seven per 100,000 population.<sup>55)</sup> There has been no confirmed case with legionellosis to our knowledge but dozens of patients probably develop legionellosis every year in Bhutan, though the prevalence of legionellosis will be affected by natural and cultural conditions in each region. In legionellosis, pneumonia is the predominant clinical manifestation but respiratory symptoms are sometimes not prominent. Clinically, several findings including presence of diarrhea, confusion, hyponatremia, renal dysfunction, hepatic dysfunction, and/or hematuria can be clues for the diagnosis but not highly specific.<sup>56-59)</sup>

Although prompt treatments with the newer macrolides or the respiratory tract quinolones are critically important, legionellosis has been overlooked in many cases. Physicians should consider the possibility of legionellosis, especially in patients with severe CAP.

One of the limitations of this study must be that we used only a rapid detection kit for *Legionella* species targeting 16S rRNA of *Legionella* species. We can't completely deny the possibility of false-positive. We have already confirmed that *Legionella pneumophila* is abundantly isolated from puddles on roads by culture method in Japan and, in this study, we tried to show an evidence that *Legionella* species exist also in Bhutan.<sup>50)</sup> The present test kit detects not only *Legionella pneumophila* but also other *Legionella* species. It has

been reported that the majority of cases with legionellosis are caused by *Legionella pneumophila*, followed by *Legionella longbeachae* and *Legionella bozemanii*. However, it is still not clear whether *Legionella pneumophila* is more pathogenic than other *Legionella* species. All *Legionella* species are considered potentially pathogenic for humans.

The climate in Bhutan is varied because of the difference in altitude from less than 100 m in the south to more than 7500 m in the north within less than 200 km aerial distance.<sup>52)</sup> The southern part has a subtropical climate which is humid and hot throughout the year. The Inner Himalayan region has a temperate climate and the extreme north has a cold tundra type. About the precipitation, it has been reported that Phuntsholing, a main city in southern Bhutan, received a mean annual precipitation of more than 4000 mm, with 800 mm in the month of July alone, whereas Khomachu, a town in northeastern Bhutan, received less than 800 mm per year.<sup>59)</sup> Monitoring the impact of legionellosis in different regions of the country may contribute to better understanding of relations between legionellosis and climate. In recent paper, we argued that legionellosis should now be added to the IPCC's list of important climate sensitive health issues.<sup>60)</sup> The detection of *Legionella* species in Bhutan may be a small but significant step to monitor and control legionellosis at a global level.

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