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The Utilization of a Commercial Soil Nucleic Acid Extraction Kit and PCR for the Detection of *Clostridium tetanus* and *Clostridium chauvoei* on Farms after Flooding in Taiwan

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ABSTRACT. Clostridial diseases are zoonoses and are classified as soil-borne diseases. *Clostridium chauvoei* and *Clostridium tetani* cause blackleg disease and tetanus, respectively. Since bacteria and spores are re-distributed by floods and then, subsequently, contaminate soils, pastures and water; the case numbers associated with clostridial diseases usually increase after floods. Because Taiwan is often affected by flood damage during the typhoon season, possible threats from these diseases are present. Thus, this study's aim is to apply a combination of a commercial nucleic acid extraction kit and PCR to assess the prevalence of *Clostridia* spp. in soil and to compare the positivity rates for farms before and after floods. The minimum amounts of *Clostridium tetanus* and *Clostridium chauvoei* that could be extracted from soils and detected by PCR were 10 and 50 colony forming units (cfu), respectively. In total, 76 samples were collected from the central and southern regions of Taiwan, which are the areas that are most frequently damaged by typhoons. Noteworthy, the positive rates for *Clostridium tetanus* and *Clostridium chauvoei* in Pingtung county after the severe floods caused by a typhoon increased significantly from 13.73 and 7.84% to 53.85 and 50.00%, respectively. This study for the first time provides the evidence from surveillance data that there are changes in the environmental distribution of *Clostridium* spp. after floods. This study indicates that screening for soil-related zoonotic pathogens is a potential strategy that may help to control these diseases.

KEY WORDS: bacterial zoonoses, diagnose, PCR.

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Various zoonotic diseases, including Clostridium and Leptospira, are also soil-borne diseases. They have been reported worldwide in Asian, African and European countries including Japan, Zambia, Nigeria and United Kingdom [2, 9, 19, 22]. *Clostridia* are widely distributed in nature and are found in soil as well as freshwater and marine sediments throughout the world. Ecological factors play a significant role in the development of clostridial soil-borne diseases, especially among those species that cause infection [9].

Clostridium chauvoei causes blackleg disease in young cattle and sheep. It is the most common clostridial disease and has a high mortality [3]. Pastures, soil, and feces are often contaminated with *Clostridium chauvoei*. Animals are usually infected with *Clostridium chauvoei* through eating silage and hay contaminated with the vegetative bacteria and spores. They may also contract the disease via an accidental puncture or when a pre-existed wound is exposed to polluted soil and water. Another acute and fatal disease that often affects cattle, sheep, and humans is caused by *Clostridium tetanus*. *Clostridium tetanus* exists in soil everywhere, and its origin may be animal excrement. The spores of *Clostridium tetanus* are highly resistant and can be transmitted by wind and water to the surrounding environment allowing colonization of new areas. When penetrating and deep wounds are exposed to *Clostridium tetanus* due to contact with soils, water or mud, the damaged tissues provide an anaerobic environment that allows the propagation of the bacteria and the production of its lethal toxin. The existence of *Clostridium tetanus* in the environment may result in either single events or sporadic outbreaks [1, 4, 7]. It is also the most fatal zoonosis during floods, and a peak of more than 100 human cases of *Clostridium tetanus* infection in a single month was reported after the large tsunami in Achei in 2005 [12]. Unlike other *Clostridium* spp. which are either ubiquitous or only contracted by ingestion of contaminated material, *Clostridium chauvoei* and *Clostridium tetanus* are significant diseases and were selected as targets for this study based on their importance, their transmission characteristics, and their relationship to natural disaster events. Recent studies in Nigeria and Zambia have indicated that floods from rivers during the rainy season seem to bring a large number of *Clostridium* spores into the soil and these then contaminate the environment. This then significantly raises the risk of clostridial diseases [2, 11]. The incidence and distribution of clostridial disease have also been shown to coincide with locations near river and stream systems in Japan and the United Kingdom [19, 22]. It is worth noting that the case numbers of clostridial diseases usually increase after a heavy rainfall. The geographic location of south-east Asia

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countries, including Taiwan, means that they are within the track of a number of typhoons every year, and flood damage due to typhoons is a common occurrence annually. Therefore, it seems likely that extreme climate events may play an important role in the promotion of clostridial diseases in Taiwan and elsewhere in the region. However, there is a lack of background information on the environmental epidemiology of clostridial diseases. To overcome this, it is necessary to establish a sensitive and rapid system for evaluating the presence of such organisms. Using a similar approach to that used for other bacteria such as *Leptospira* spp., the extraction of environmental nucleic acid from soils by a direct method is one possible approach [15]. Compared to indirect methods, a large amount of nucleic acid can be obtained by extracting the DNA of lysed bacteria from soil. However, inhibitory substances, including fats, humic acids, proteins, and volatile fatty acids, are inevitably co-purified with DNA when this method is used and the protocol used needs to be modified in order to neutralize their effect [16, 17]. Recently, several commercial kits for the extraction of soil DNA have become available, and these have the advantages of a quick turn-around time and an easy standard procedure [14]. To our knowledge, no application of such a DNA extraction kit and PCR has been applied to soil containing clostridial soil-borne disease. This study has the aim of investigating the utilization of a commercial soil nucleic acid extraction kit combined with PCR for the detection of *Clostridium chauvoei* and *Clostridium tetanus* and comparing the sample positive rates before and after floods. The results will help to design screening strategies for the detection of these pathogenic bacteria and other soil-related zoonotic pathogens in the future.

MATERIALS AND METHODS

Background information and collection of soil samples: During August 2009, typhoon Morakot struck Taiwan, and this resulted in severe floods in the southern Taiwan including the areas around Kaohsiung, Pingtung and Taitung. The total rainfall in both flat and mountainous areas ranged from 1,000 to 2,000 mm per day. The resulting massive damage drew an immediate reaction from the Taiwan government and as a result of this typhoon, a preventive surveillance program was set up to provide a baseline in the aftermath of the floods. Across a number of farms, approximate fifty grams of soil from a depth of 10 cm was gathered separately from three locations, namely close to the farm house, intermediate from farm house, and distant from the farm house [9]. These samples were collected from twenty-one dairy farms in Miaoli, Taichung, Yunlin, Chiayi, Kaoshiung, Pingtung and Taitung counties. The sampling positions are in the central and southern regions of Taiwan. Most cattle farms in Taiwan are clustered in these areas, and these farms frequently receive seasonal floods due to typhoons. To establish the baseline dataset, soil samples were collected during May 2010 and surveyed for the presence of DNA from *Clostridium chauvoei* and *Clostridium tetanus*. Any farm on which one sample tested positive was regarded as a positive farm. Most important to this study, typhoon Fanpai affected Taiwan during September 2010, and there was a massive rainfall in both Kaoshiung and Pingtung counties. After the floods, soil samples from the same farms were collected and screened; these results formed the after flood dataset. The positive rates between the before flood and after flood datasets were compared to identify changes in the environmental distribution of *Clostridium* spp. after the flooding caused by the typhoons, and results were analyzed using the χ^2 test. A value of $P<0.05$ was considered to be significant. In total, 76 samples were processed to detect *Clostridium chauvoei* and *Clostridium tetanus* in parallel.

Soil DNA extraction: The SoilMasterTM DNA Extraction Kit (EPICENTRE® Biotechnologies, PA, U.S.A.) was used for the extraction and subsequent purification of the environmental DNA from soil samples following the manufacturer's instructions. A hundred mg of soil samples was mixed with 250 μl of soil DNA extraction buffer and 100 μg of proteinase K. The mixture was vigorously shaken at 37°C for 10 min to raise the yield of DNA. To lyse spores within the sample, 50 μl of lysis buffer was added, and samples were incubated at 65°C. After centrifugation and the addition of a precipitation reagent, proteins were precipitated on ice for 8 min and then removed by centrifugation. To remove inhibitory substances, the supernatant was passed through a spin column, and the DNA was then precipitated using DNA precipitation buffer at room temperature for 5 min. After the DNA had been centrifuged down, the resulting precipitant was washed with 500 μl of wash buffer and finally the DNA was dissolved in 20 μl of Tris-EDTA buffer.

Detection limit for Clostridium chauvoei and Clostridium tetanus: To determine the minimum amount of *Clostridium tetanus* and *Clostridium chauvoei* DNA that can be detected, fresh pure cultures of *Clostridium tetanus* and *Clostridium chauvoei* were streaked on blood agar plates using the surface spreading technique. Colonies were picked from plates using a sterile swab. Direct colony suspensions were made in normal saline, and their turbidities were adjusted into 0.5 McFarland standards. The suspensions were further subjected to ten-fold serial dilutions. One milliliter of diluents was inoculated on blood agar plate and then incubated in an anaerobic jar at 37°C. After 2 days, the viable plate count method was used for the enumeration of bacteria. The results were presented as colony forming units (cfu/m*l*). The same amounts of diluents were pre-mixed with 1 g of soil sample that did not contain either *Clostridium tetanus* or *Clostridium chauvoei*. A hundred mg of these samples was then subjected to the DNA extraction followed by PCR.

PCR for the detection of Clostridium spp.: In order to detect *Clostridium tetanus* and *Clostridium chauvoei* DNA in the extracted soil DNA, PCR was used to target the tetanus toxin gene and the 16S rRNA sequence as described by Kuhnert *et al.* (1997) and Cordoba *et al.* (2011), respectively. The PCR primer sets for *Clostridium tetanus* and *Clostridium chauvoei* were *Clostridium tetanus*-Tet 1 and *Clostridium tetanus*-Tet 2 and *Clostridium chauvoei*-16S-L and *Clostridium chauvoei*-193-R, respectively (Table 1). The expected sizes of the PCR products for *Clostridium tetanus* and *Clos-*

Table 1. The primers used for the detection of the toxin gene from *Clostridium tetanus* and the 16S rRNA gene from *Clostridium chauvoei*

Primer name	Type	Sequences (5' –3')	Nucleotide position
Clostridium chauvoei-16S-L	Forward outer	GTCGAGCGAGGAGAGTTC	nt. 61–82 in *E. coli* number [13]
Clostridium chauvoei-193-R	Reverse outer	CGGATTGCTCCTTTAATTAC	nt. 193–220 in *E. coli* number [20]
Clostridium tetani-Tet 1	Forward	CCTAGTTTCAAAACTTATTGGCTTATGTAA	nt. 1671–1700 in X06214 [6]
Clostridium tetani-Tet 2	Reverse	CATAATTCTCCTCCTAAATCTGTTAATGAT	nt. 1991–2021 in X06214 [6]

tridium chauvoei were 160 and 350 bp, respectively. A 25 μl PCR reaction mixture, which consisted of 0.2 mM of each dNTP, 2.0 mM $MgCl_2$, 0.2 μl of Taq polymerase (5 units/μl) and 0.1 μM of each primer, was used. An appropriate amount of extracted soil DNA was used to examine the presence of *Clostridium tetanus* and *Clostridium chauvoei* DNA in the soil samples. PCR was initiated at 94°C for 5 min, and this was then followed by 35 cycles of denaturation at 94°C for 45 sec, annealing at 57°C (for *Clostridium chauvoei*) or 55°C (for *Clostridium tetanus*) for 45 sec and extension at 72°C for 1 min. The PCR reaction was completed with a final extension at 72°C for 10 min. The PCR products were analyzed on a 2% agarose gel/TAE buffer containing 0.5 μg/m*l* of ethidium bromide. PCR products of the expected sizes were then purified and sequenced using the appropriate primers in order to confirm their authenticity.

RESULTS

The geographic locations of sampling farms are shown in Fig. 1. They are located in the central and southern regions of Taiwan. The northern region of Taiwan was not covered in this screening program, because few dairy farms are located in the northern part of Taiwan and this area is less prone to typhoons.

Different amounts of *Clostridium tetanus* and *Clostridium chauvoei* were mixed with soil samples, and their DNA was extracted and amplified using PCR. For the *Clostridium tetanus* DNA, the yields of product decreased in a dose-dependent manner relative to the amount of bacteria present. The detection limit was 10 cfu/g (Fig. 2A). The effect of the background DNA in the soil samples on the amplification of plasmid DNA was also measured, and the lowest amount of plasmid that could be detected was 1 copy/g (Fig. 2B). This indicated that the PCR efficiency for genomic DNA was 10-fold lower than that for plasmid DNA. This process was repeated for *Clostridium chauvoei*, and the minimum amount of bacteria in soil sample that could be detected was 50 cfu/g (Fig. 3A). Similarly, the detection limit for plasmid DNA in the presence of environmental DNA was 1 copy/g (Fig. 3B). The specificity of these PCR products was confirmed by sequencing.

When the prevalence of *Clostridium tetanus* at baseline was determined, no positive samples were collected from farms in Yunlin, Chiayi and Kaoshiung counties, and the positive rates for farms from Miaoli, Taichung, Pingtung and Taitung counties were 28.57, 66.67, 13.73 and 33.33%, respectively. After the rain from Typhoon Fanpai, a significant reduction in the number of positive farms in Miaoli

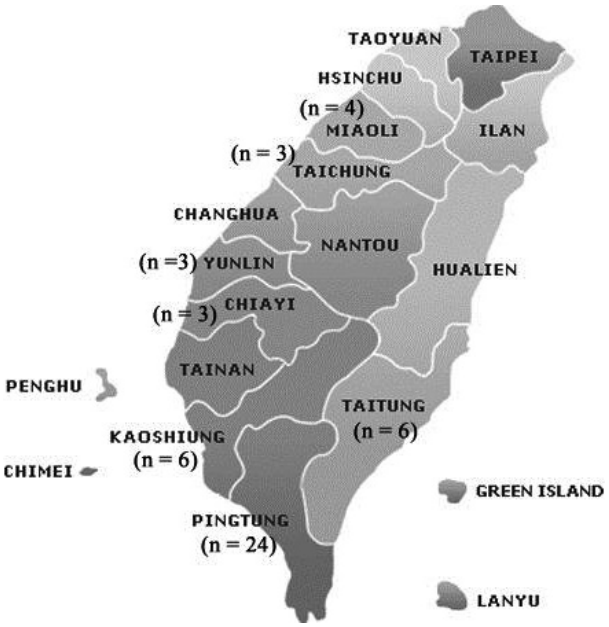

Fig. 1. Map showing the location of the sampled cattle farms and their number in each area of Taiwan. *Number of sampled cattle farms.

county (28.57 vs. 15.38%; $P<0.05$), Taichung county (66.67 vs. 15.38%; $P<0.05$) and Taitung county (33.33 vs. 15.38%; $P<0.05$) was found. In contrast, a significant increase in the number of positive farms in Pingtung county was observed (13.73 vs. 53.85%; $P<0.05$) (Fig. 4). Similar baseline results for *Clostridium chauvoei* were found, with the soil samples from Yunlin, Chiayi and Kaoshiung counties being all negative. While a marginal difference was observed at Miaoli county (14.29 vs. 12.50%; $P>0.05$), slightly but no significantly higher difference was found in Taitung county (33.33 vs. 25.00%; $P>0.05$). The positive rate was significantly reduced after the floods in Taichung county (33.33 vs. 12.50%; $P<0.05$), while the positive rate was significantly increased in Pingtung county (7.84 vs. 50.00%; $P<0.05$) (Fig. 5). Therefore, the highest prevalence of *Clostridium tetanus* and *Clostridium chauvoei* after the floods was found to be in the soils from Pingtung county for both pathogens and in both cases there had been a significant increase in the positive rate after the floods compared to before the floods.

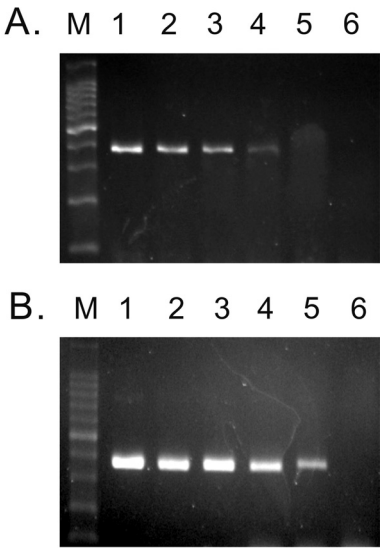

Fig. 2. The detection limit for *Clostridium tetanus* (A) using soil DNA and PCR. M: marker, lanes 1–5 (10^4, 10^3, 10^2, 10 and 1 cfu/g) and lane 6: negative control. The detection limit for the pGEM-Teasy-Tet plasmid containing the *Clostridium tetanus* toxin gene (B) using soil DNA and PCR. M: marker, lanes 1–5 (10^4, 10^3, 10^2, 10 and 1 copies of plasmids/g) and lane 6: negative control.

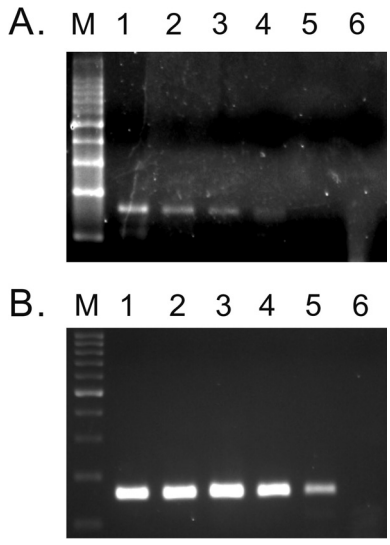

Fig. 3. The detection limit for *Clostridium chauvoei* (A) using soil DNA and PCR. M: marker, lanes 1–5 (5×10^4, 5×10^3, 5×10^2, 50 and 5 cfu/g) and lane 6: negative control. The detection limit for the pGEM-Teasy-SLR plasmid containing the *Clostridium chauvoei* 16S gene (B) using soil DNA and PCR. M: marker, lanes 1–5 (10^4, 10^3, 10^2, 10 and 1 copies of plasmids/g) and lane 6: negative control.

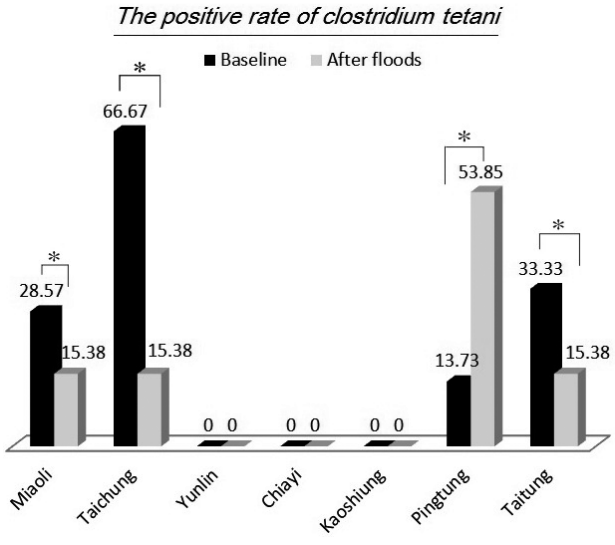

Fig. 4. The prevalence of *Clostridium tetanus* across the various different counties of Taiwan. Both the positive rates at baseline before flooding and the positive rates after floods are listed and compared using the χ^2 test. * indicates the significant difference (P<0.05).

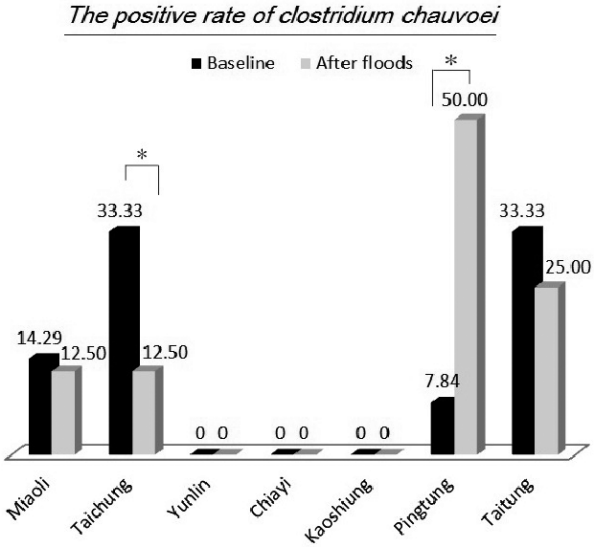

Fig. 5. The prevalence of *Clostridium chauvoei* across the various different counties of Taiwan. Both the positive rates at baseline before flooding and the positive rates after floods are listed and compared using the χ^2 test. * indicates the significant difference (P<0.05).

DISCUSSION

An absolutely anaerobic culture is necessary for the isolation of *Clostridium chauvoei* and *Clostridium tetanus*, which is both time-consuming and expensive; as a result of this, the standard approach to cultivating these organisms is difficult to implement and is often impeded by the outgrowth of other contaminating anaerobic bacteria that exist in normal soil flora [18]. Bacterial endospores are resistant to harsh environments such as the digestive system of animals,

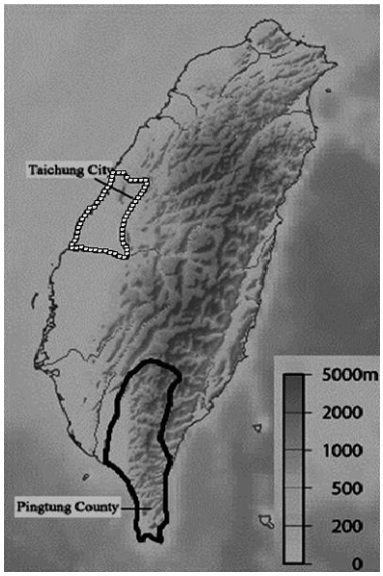

Fig. 6. The topology of Taiwan. The grey scales represent the average altitude of the land. The areas encompassing Taichung and Pingtung counties are outlined in blue and red lines, respectively.

and as a result, both vegetative bacteria and spores can be shed into the pasture and soil. An enrichment step during the isolation of *Clostridium perfringens* and *Clostridium tetanus* can increase the population of bacteria and improve the detection rate by PCR; nevertheless, the main challenge remains the purification of *Clostridium* spp. without interference from other anaerobic flora, especially when the soil sample is highly contaminated. Therefore, the bacterial counts obtained by culture are likely not to truly reflect the real count in the soil sample and may overestimate the risk of disease [8]. In such circumstances, the direct extraction of DNA from soils and subsequent PCR are likely to more truly reflect the presence of pathogens in a soil sample, and this approach was used in the current study. Recent studies have indicated that soil provides a good environment for the survival of *Clostridium chauvoei* in Nigeria, Sweden and Zambia, which had high prevalence of clostridial infections [2, 3, 9]. In these circumstances, the detection of *Clostridium* spp. in soil samples is a good approach to obtaining a reliable risk evaluation for both animals and humans.

Although a specific bacterial DNA from bacteria in soil samples can be easily amplified using PCR, it is still necessary to reduce interferences from inhibitors that are co-purified with the background DNA by either diluting the extracted DNA or using resins provided by a commercial kit to remove inhibitors. Since the efficiency of PCR is likely to deteriorate because of over-dilution, only the purification of DNA by resins was used in this study [10]. In a previous study, it was indicated that the minimum amount of bacterial DNA from samples containing soil, silage, and manure that can be detected using PCR was about 200 cfu/g [3]. However, the detection level was found to approach 10 cfu/g when cleaner samples with less inhibitory substances were amplified [18]. The detection levels of DNA from soils using PCR for *Clostridium tetanus* and *Clostridium chauvoei* in this study were both lower than 200 cfu/g at 10 and 50 cfu/g, respectively. Based on the above, these seem to be acceptable detection limits. In addition, when we tested the detection limits using two plasmids carrying genes from either *Clostridium tetanus* or *Clostridium chauvoei*, the detection limits were around 10-fold higher than those for genomic DNA. This may be explained by the presence of large amount of contiguous non-target DNA with a higher complexity in the bacterial samples, which is likely to change binding probability of the primers and lead to a lower availability of target sequences during amplification.

Soil-borne diseases are a persistent problem that is often neglected across livestock farms in Taiwan. This can be primarily attributed to a shortage of field records and the absence of epidemiological surveillance programs. The latter is partly due to a lack of feasible techniques that are both reliable and affordable. The fact that any approach used needs to be supported financially by public services may be a significant problem. In this context, we used soil samples collected from the major animal husbandry areas of Taiwan to address the problem of soil borne disease, and the current study offers firsthand information on running such an appropriate surveillance program for *Clostridium* spp. The average positive rates for *Clostridium tetanus* and *Clostridium chauvoei* on the farms at baseline before flooding were all less than 23.32%. This should be compared to the range 30.00 to 42.00%, which covers the average positive rates for *Clostridium* spp. in the soil worldwide. Thus, in general, the contamination rates for *Clostridium tetanus* and *Clostridium chauvoei* in Taiwan would seem to be somewhat lower than those found in other countries [21]. There are three possible explanations. First, if Sweden, Zambia and Nigeria are examined, these countries have more extensive livestock production systems than Taiwan. Unlike Taiwan, blackleg disease is a major endemic disease in those countries. It seems likely that a lower density of cattle in Taiwan results in fewer shedding sources for *Clostridium* spp. Second, compared to Taiwan, the climate and location of cattle farms in Zambia and Nigeria are more favorable for the development of clostridial diseases. There is a long rainy season in Zambia and Nigeria every year, and during this season, the river flats of Zambia and Nigeria are usually flooded. As a result, large amounts of *Clostridium* spp. spores are spread to the soil of farms near rivers. However, most cattle farms in Taiwan are not near a river, and there are only sporadic floods, which are brought out by typhoons. Third, our current sampling numbers are less than those from studies in other countries, and it is possible that an increase in positive rates for farms would occur if more samples are included. Taken together, the above factors play a significant role in the lower positive rates for farms at baseline before flooding compared to other countries.

However, the profoundest changes in the positive rates between the baseline and after floods were observed in Tai-

chung and Pingtung counties. These all occurred after the same flooding event that was generated by typhoon Fanpai. Considering the hydro-geophysical characteristics of Taiwan as shown in Fig. 6, since the cattle farms in Taichung county are located on the plains, it is clear that heavy rainfall may wash out environmental *Clostridium* spp. from the soil and their physical redistribution may have led to a lower positive rate for the farms in this area. However, this was not the case in Pingtung county. A massive amount of rainfall on the hilly and mountainous areas caused significant flooding. In these circumstances, it is possible that congested water drainage resulted in the concentration of sediment containing bacteria and spores on the sampled low level farms located in this area. Sequencing of the PCR products from the *Clostridium* DNA in the soil DNA samples showed that the sequences were identical to each other even though the soil samples came from different farms, which were located across the whole flood-affected area of Pingtung county. This implies that the bacteria came from the same source. The longest straight-line distance between the Pingtung farms is less than 60 km, and it is likely that the same flood event caused the redistribution of the bacteria across all of the farms. The fact that it is possible to identify relationships between sources of pathogenic microorganism in these circumstances is an additional advantage of the present approach. Although no field records enumerating *Clostridium* spp. infections in animals are available at this point, a recent report has indicated the case number of *Clostridium tetanus* in human after the floods increased about three-fold compared to before the floods [5]. This increase in number of *Clostridium* tetanus infection cases was found in the southern Taiwan, which is the same area that has the highest prevalence of *Clostridium tetanus* and *Clostridium chauvoei* after the floods in this study [5]. Additionally, in a manner that paralleled the present results, an outbreak of another soil-bored disease, *Leptospirosis*, has been laid at the door of the severe floods generated by the Morakot typhoon, and this event affected 203 individuals. Most of these infected individuals were also from Pingtung county [5].

In summary, this commercial soil extraction kit combined with PCR for the detection of soil-based *Clostridium* spp. was proven to be a useful approach, and results indicated the positive rates for *Clostridium tetanus* and *Clostridium chauvoei* in Pingtung county, the south part of Taiwan, after the severe floods caused by a typhoon were increased to above 50.00%. This approach can be used as a part of a national surveillance program associated with major weather and climate change events. Apart from acquiring epidemiological information that should help to implement rapid quarantine responses, such up-to-date information should also help the government to make better economic and correctly targeted choices about vaccine programs, especially after a natural disaster [9]. Since *Clostridium* spp. are organisms that cause various zoonoses, this information will also be useful to farmers, veterinarians, and service industry personnel in the affected areas, who then can be cautious when dealing with corpses, removing waste material and working with damp soil/mud. Increased care will help to avoid accidental contact with bacteria or spores, and this will decrease morbidity. Finally, an ongoing study that focuses on an epidemiological analysis of sick animals and humans is needed, before a definitive relationship between clostridial diseases and the prevalence of *Clostridium* spp. in soil samples, especially after the floods in Taiwan, can be conclusively proved.

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