Original papers

Detection of *Cryptosporidium parvum* DNA in fecal samples of infected cattle (*Bos indicus*) and water buffaloes (*Bubalus bubalis*) in the Philippines using loop-mediated isothermal amplification method

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ABSTRACT. Fecal DNA samples from 17 cattle and 38 water buffaloes found to be infected with *Cryptosporidium* oocysts using Kinyoun acid fast stain from a previous study, were subjected to loop-mediated isothermal amplification (LAMP) assay using specific primers for *Cryptosporidium parvum* (*C. parvum*) from three municipalities, Maria, Baler and San Luis of the province of Aurora in the Philippines. Results of the fecalysis using Kinyoun acid fast stain and LAMP assay were compared with the PCR results of the examined farmer/owner who raised these animals to determine the possible zoonoses of *C. parvum* between the farmers and their animals. Using LAMP assay, only 41% (7/17) were positive in cattle and 76% (29/38) in water buffaloes. Out of the seven LAMP positive cases in cattle, 86% (6/7) came from Maria and 14% (1/7) from Baler. Out of 29 LAMP positive cases in water buffaloes, 62% (18/29) came from Maria, 24% (7/29) from Baler and 14% (4/29) from San Luis. Comparing with the earlier results for probable zoonoses of *C. parvum* between the farmers and their animal was determined. Eight farmers that were positive in PCR and with their water buffaloes, positive in LAMP assay were detected to have *C. parvum*. Only one farmer with his cattle was detected positive of *Cryptosporidium* spp. in PCR, however, it was negative in LAMP assay hence, a non-*parvum* species might infected the farmer and the animal.

Key words: *Cryptosporidium parvum*, cattle, water buffalo, LAMP

Introduction

Cryptosporidiosis, recognized as a major public health concern affecting both humans and animals, is caused by a microscopic parasite of the genus *Cryptosporidium*. The common occurrence of zoonotic subtypes of *Cryptosporidium parvum* (*C. parvum*) in cattle [1–3] and water buffaloes [4,5] highlights the potential role of these animals as significant reservoirs of infection and can be transmitted to humans becoming a serious public health concern [6].

Once an animal or human is infected, sporulation occurs within the host and passed out as infective oocyst in the feces contaminating the soil and surface water. Cryptosporidiosis is clinically characterized by gastrointestinal disorders and less commonly by the respiratory, liver and pancreatic disorders. It produces large economic losses, especially when the etiologic agent is mainly involved in triggering neonatal diarrhea [7].

Based on a survey of 7,369 calves from 1,103 dairy farms located in 28 states [8], found that more than 50% of 2-week-old calves and 22.4% of all

Fig. 1. Map of the Aurora Province in the Philippines. Red dots indicates the municipalities (Maria, Baler and San Luis) where the samples were collected.

calves (ages, 1 to 17 weeks) tested positive for *C. parvum*. This concluded that virtually all herds with more than 100 cows are infected with *C. parvum*. Limited data suggest that adult cows may also shed oocysts. Scott et al. [9] found up to 18,000 oocysts per gram of feces from apparently healthy adult cows.

In the Philippines, the first documented case of both human and water buffalo (*Bubalus bubalis*) infection was reported in Palawan, Philippines [10]. A total of 831 individuals were queried; 41 with diarrhea, 19 of those returned stool specimens. Domestic animal stools were screened for *Cryptosporidium*. One cattle and one water buffalo were found positive. One patient, a 5-year-old male, was infected. The family dwelling was inspected and found to be a bamboo thatch structure elevated on stilts where a variety of domestic livestock including pigs, dogs, cats, chickens and water buffaloes, all documented hosts of *Cryptosporidium* directly around and frequently under the house. Although the exact mode of transmission of oocysts is unknown, it would again be reasonable to assume that in this community human-animal contact with subsequent hand-to-mouth transfer of oocysts is a potential source of infection [10].

During the past decade, fecal samples stained with Kinyoun acid fast were specific for

demonstrating the oocysts of *Cryptosporidium*. However, by oocyst morphology alone with little or no host specificity and/or molecular data to support identification, problems on recognizing how many reported isolates are actually there for *C. parvum* or other species arise.

Domingo [11] reported the presence of *C. parvum* detected in the fecal samples of small holder farmers in three selected municipalities of Aurora province using PCR with a prevalence of 21% (142/678). PCR was used to specifically amplify the DNA of *C. parvum* in the fecal DNA samples of the farmers. Similarly, Domingo also reported a prevalence of 29.6% (63/212) in cattle and 21.1% (49/231) in water buffaloes using microscopy after staining the fecal samples with Kinyoun acid fast. Kinyoun stain is specific for *Cryptosporidium* oocysts but does not identify the species.

Experimental studies of both humans and animals suggest that diverse species and genotypes show different levels of infectivity and virulence. Others have pointed out those zoonotic strains of *C. parvum* produce more severe infections in humans than the strains found only in humans. Moreover, the potential reservoir hosts and transmission pathways for the novel species infecting humans are unclear.

A new specific DNA amplification technique called loop-mediated isothermal amplification (LAMP) was developed [12] and has already been applied to the detection of pathogenic viruses [13], bacteria [14], parasites [15], fungi [16] and *Cryptosporidium* [17].

LAMP, which does not involve thermal cycling as done in Polymerase Chain Reaction, allows the rapid amplification of DNA with high specificity under isothermal conditions using DNA polymerase with strand-displacement activity. Specific nucleotide extension is achieved with four primers recognizing six distinct regions on the target. The amplification time can be further shortened by using an additional two primers, termed the loop primers [18]. Moreover, LAMP products can be detected not only using specialized equipment but also by visual observation of turbidity or fluorescence [19].

Cryptosporidium infection in animals and humans is known to produce different levels of infectivity and severity and even different responses to treatment. The main causative agents of human cryptosporidiosis are strains of human and bovine genotypes of the species *C. parvum* (also called

genotype 1 and genotype 2, respectively). Previous reports presented 21% (142/678) prevalence of *C. parvum* among small holder farmers in three selected municipalities of Aurora province using PCR [11] while 5% in a communal water buffalo farm [20]. On the other hand, *Cryptosporidium* oocysts were found in cattle and swamp water buffaloes with prevalence of 26.9% (57/212) in cattle and 21.2% (49/231) in water buffaloes. However, the main species of *Cryptosporidium* present in cattle and swamp water buffaloes was not identified.

This study identified *C. parvum* in the fecal DNA of cattle and swamp water buffaloes found to be infected with *Cryptosporidium* oocysts using LAMP. Results of the study clarified the potential role of the animal in the transmission of the genotype of *C. parvum* infecting small holder farmers exposed to cattle and swamp water buffaloes which were infected with cryptosporidiosis.

The objective of this study was to identify DNA of *C. parvum* in the fecal samples of cattle and water buffaloes infected with cryptosporidiosis using LAMP assay.

Materials and Methods

Samples used. The samples used were feces of 17 cattle and 38 swamp water buffaloes collected from Baler, Maria Aurora and San Luis in Aurora province (Fig. 1) that were previously tested and found positive of *Cryptosporidium* oocyst using Kinyoun stain [11]. These samples were placed in a microcentrifuge tube with 10% formalin and processed further for DNA extraction.

DNA extraction. Genomic DNA extraction was done from sediments of preserved cattle and swamp water buffalo fecal samples. The pellets resulting from the sedimentation procedure were washed three times with phosphate buffer solution (PBS). The pellets from the last wash were resuspended in 200 µl TE buffer (10 mM Tris, ph 7.4 and 1 mM EDTA, pH 8.0). Two hundred µl lysis buffer (Tris, $Na_2EDTA.2H_2O$, NaCl, SDS and sterile distilled water) was added followed by 8 µl of proteinase K (50 mg/ml). The resuspended pellets were incubated at 60°C for one hour; after which, an equal volume of phenol, chloroform and iso-amyl mixture (PCI) was added. The tube was placed in a shaker for 30 min to mix the contents and then centrifuged at 13,000 rpm for 4 min at 10°C. The aqueous phase was transferred into a new microcentrifuge tube (MCT) and an equal volume of PCI was again

added. The tube was inverted for 5 min; then the tubes were centrifuged at 13,000 rpm for 4 min at 10°C. The aqueous phase was transferred to a new MCT. The resulting DNA that stuck to the tube was precipitated with a thawed preparation of 3 M frozen sodium acetate at 1:10 ratio followed by chilled absolute ethanol at 2:1 ratio. The tube was kept at -20°C overnight. The following day, the tube was centrifuged at 12,000 rpm for 10 min at 4°C. The supernatant was discarded and 1 µl of 70% ethanol was then added into the tube. It was centrifuged at 12,000 rpm for 5 min at 4°C. The supernatant was discarded and the sediments left inside the tube were air dried by inverting the tube on a paper towel to drip excess liquid. The resulting DNA which appeared as string-like material stuck on the sides of the tube was rehydrated with 20 µl of 10 mM TRIS (pH 7.4)/1 mM EDTA (TE buffer) and stored at -20°C until use [21,22].

LAMP DNA Oligonucleotide Primers. The LAMP primer set used, was designed from the 60 kD glycoprotein gene (gp60) of *C. parvum* (Accession no: AB237136) in Japan. The LAMP primer sequences were as follows: F3: 5'-TCG CAC CAG CAA ATA AGG C-'3; B3: 5'-GCC GCA TTC TTC TTT TGG AG-'3; FIP: 5'-ACC CTG GCT ACC AGA AGC TTC AGA ACT GGA GAC GCA GAA-'3; BIP: 5'-GGC CAA ACT AGT GCT GCT TCC CGT TTC GGT AGT TGC GCC TT-'3. Table 1 shows the LAMP primer mixture used in the study.

Optimization of LAMP Assay. Lyophilized DNA isolate of *C. parvum* was provided by the National Research Center for Protozoan Diseases at Obihiro University, Japan. Ability of LAMP to detect *C. parvum* was assessed using the three indicators: fluorescent test, dye test and gel electrophoresis test.

For the determination of optimal condition, LAMP reaction was performed in a total volume of 12.5 µl containing 4.5 µl of sterile double distilled water, 3.0 µl of 5 M Betaine (Sigma), 1.5 µl of 10X Thermopol Buffer (New England Biolabs), 1.0 µl of 2.5 mM each dNTP (Intron), 0.75 µl of primer mix, 0.75 µl of Bst Polymerase (New England Biolabs) and 1.0 µl of DNA template. Sterile water was used as a negative control template. The mixture was incubated at different temperatures from 60°C up to 65°C for 60 min in a heat block [11].

LAMP Assay results. After incubation, the tubes were inspected through the naked eye for positive visible results. Positive results/indications
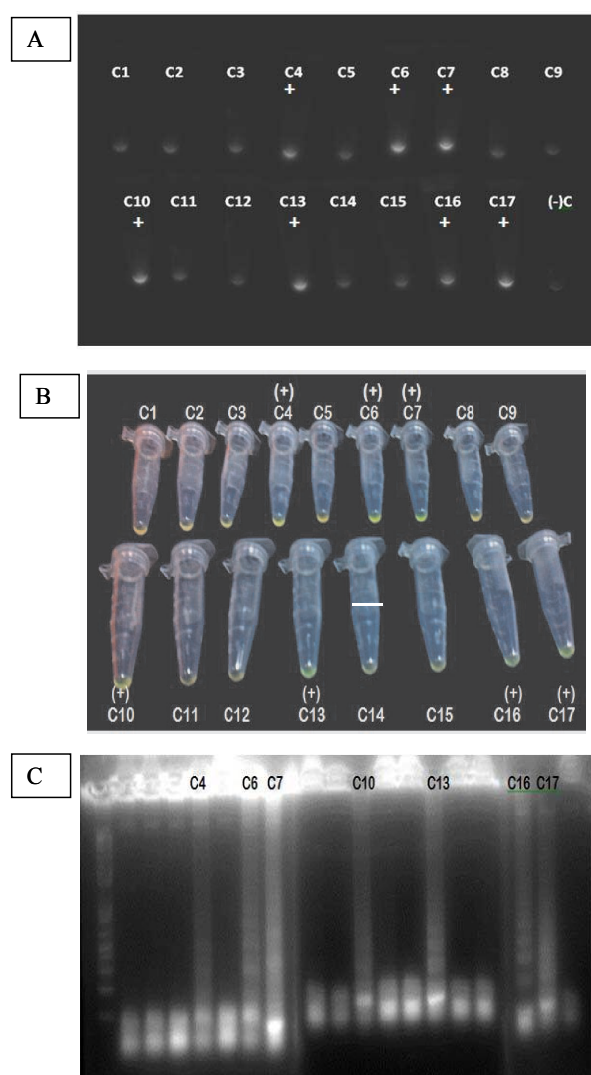
Fig. 2. Cattle samples LAMP results using different visual tools: A. Fluorescent test; B. Dye test; C. Gel electrophoresis exhibited by multiple bands

of amplification are: observation of fluorescence by placing the tubes under the UV transilluminator, color change from orange to green following the addition of 0.5 µl of SYBR green dye to the tube, seen under a natural light as well as under the UV light, and the presence of characteristic ladder of multiple bands upon visualization of the LAMP product after gel electrophoresis on a 2% agarose gel [11].

A total of 38 swamp water buffalo and 17 cattle DNA extracts were evaluated for the presence of *C. parvum*. For each fecal DNA extract, two replicates were prepared. In a 1.5 ml microcentrifuge tube, the LAMP mixture was prepared and incubated following the optimized LAMP protocol. The results were analyzed by visual inspection using the fluorescent test, dye test and gel electrophoresis test.

Positive visible reactions in the fluorescent test, dye test, and gel electrophoresis were scored in the following manner: 0=absent; 1=present. A comparison between the results of the study conducted by Domingo [11] using Kinyoun stain in water buffaloes and cattle and PCR in humans were compared with the LAMP results of water buffalo and cattle fecal DNA samples in this study. Positive samples in LAMP assay and PCR were interpreted as zoonotic genotypes since the tests specifically amplified *C. parvum* DNA, while negatives samples from both tests were marked as non-zoonotic genotypes.

Data analysis. For the evaluation of LAMP, the mode was computed to evaluate the positive results of the fluorescent test, dye test, and gel electrophoresis test. Meanwhile, the frequency of zoonotic and non-zoonotic genotype of *C. parvum* was computed according to municipality where the cattle and water buffaloes came from.

Results and Discussion

Optimization of LAMP products

After six trials, the optimum condition for LAMP test which could amplify *C. parvum* DNA was determined at 63°C for 60 min in heat block. These conditions were chosen because of fluorescence under UV transilluminator, green coloration after addition of SYBR green dye and the presence of multiple bands or ladder-like pattern of bands by gel electrophoresis.

Frequency of distribution of *Cryptosporidium parvum* cases according to source

Formalin-preserved 17 cattle and 38 swamp water buffaloes fecal samples that were positive for *Cryptosporidium* spp. oocysts using Kinyoun Acid Fast stain were used in the study. DNA from these infected fecal samples were extracted and subjected to the optimized LAMP assay using specific *C. parvum* primers. Indicators for positive results were fluorescent test, dye test, and gel electrophoresis test. Frequency distribution by municipality of infected animals that showed positive reactions in LAMP assay was determined. Detection of zoonotic and non-zoonotic *Cryptosporidium* isolates based on results from LAMP assay of animal fecal samples and PCR assay of human fecal samples was also done.

Figure 2 showed cattle fecal samples that were positive in all LAMP assay. Forty one percent or 7

Table 1. Frequency distribution of zoonotic and non-zoonotic *Cryptosporidium* cases in farmers and their cattle

Test result	Cattle		Farmer
	Kinyoun stain	LAMP assay	PCR
Positive	17 (100%)	7 (41%)	1 (6%)
Negative	0	10 (59%)	16 (94%)
Total	17	17	17

out of 17 cattle scored "1" in fluorescent test, dye test, and gel electrophoresis test. Figure 3 showed water buffalo fecal samples that were positive in all LAMP assay. Twenty nine out of 38 (76%) of water buffaloes scored "1" in dye test and gel electro-phoresis test.

DNA isolate of *C. parvum* fluoresced under the UV transilluminator giving off a bright white color as shown in Figure 2A and 2B. During DNA amplification, the by-product pyrophosphate ions are generated which bind and remove manganese ions from calcein to irradiate fluorescence. The fluorescence is further intensified as calcein combines with magnesium ions. From this feature, the presence of fluorescence can indicate the presence of target gene and visual detection can be achieved [11]. As expected, the negative control in cattle and water buffalo did not fluoresce at all.

The frequency distribution (%) of infected cattle and water buffaloes by municipality are shown in Figure 4, respectively. Out of the seven cases in cattle, 86% (6/7) came from Maria and 14% (1/7) from Baler. Out of 29 cases in water buffaloes, 62% (18/29) came from Maria Aurora, 24% (7/29) from Baler and 14% (4/29) from San Luis. The results suggest that Maria Aurora has the highest prevalence among the three municipalities. Possible reasons could be the presence of large population of cattle and water buffaloes in Maria Aurora and that bodies of water are more extensive in the municipality. *Cryptosporidium* is water borne and ingestion of oocyst contaminated water by animals is the primary means of transmission.

Zoonotic and non-zoonotic cases

Previous study reported 21% (142/678) farmers to be infected with *C. parvum* in their stools by PCR [11]. Stool samples of their cattle and water buffaloes were examined using Kinyoun acid fast stain and were detected for *Cryptosporidium* oocysts only. These fecal samples were subjected further to LAMP assay to verify the specific *C. parvum* and to determine possible zoonoses.

The percentage distribution of zoonotic cases

shared by farmers and their infected cattle detected by both PCR and LAMP assays that used specific *C. parvum* primers is shown in Table 1. Results showed that *C. parvum* infection was detected in one farmer using PCR. However, his animal was negative for *C. parvum* in LAMP despite of being detected for *Cryptosporidium* oocysts in Kinyoun stain. It may suggest that the *C. parvum* detected in the farmer could be genotype 1, the strain responsible for most human *Cryptosporidium* infections [23]. Also, the infected farmer could have been infected, not directly from his animal, but by other means such as oocyst ingestion from contaminated water since *Cryptosporidium* transmission is waterborne. On the other hand, 59% (10/17) of cattle was not detected for *C. parvum* by LAMP assay even if these animals were positive in Kinyoun stain. This implies that the other oocysts
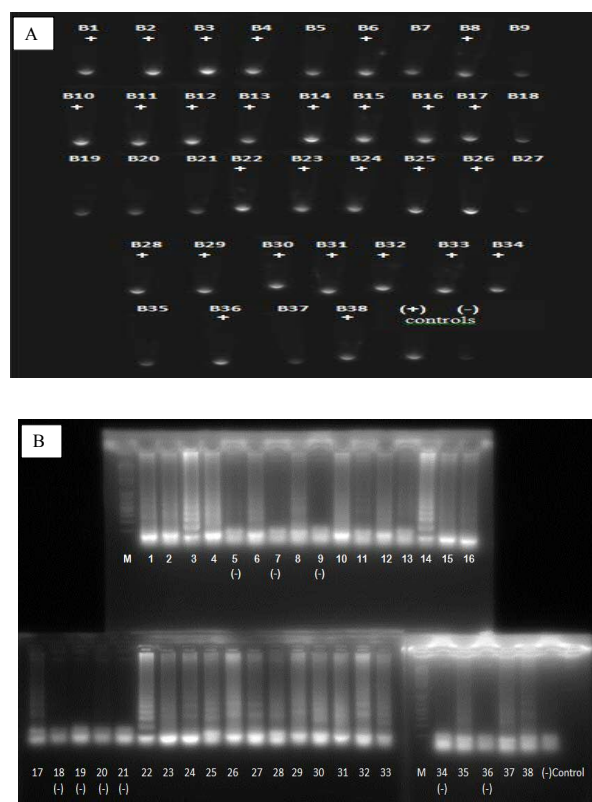

Fig. 3. A. Dye test; B. Gel electrophoresis in water buffaloes

Table 2. Frequency distribution of zoonotic and non-zoonotic *Cryptosporidium* cases in farmers and their buffaloes

Test result	Buffaloes		Farmers
	Kinyoun stain	LAMP assay	PCR
Positive	38 (100%)	29 (76%)	8 (21%) zoonotic
Negative	0	9 (24%)	30 (79%)
Total	38	38	38

detected by Kinyoun stain could be other *Cryptosporidium* species.

The percentage distribution of zoonotic cases shared by farmers and their infected water buffaloes detected by both PCR and LAMP assays that used specific *C. parvum* primers is shown in Table 2. Results showed that *C. parvum* infection was shared by eight farmers and their water buffaloes as detected by LAMP assay and PCR. This implies that the *C. parvum* present in swamp water buffalo was the genotype 2, known to largely cause human infection through contamination of water, food, or direct contact with infected animals, especially in rural areas. On the other hand, 24% (9/38) of water

buffaloes were not detected for *C. parvum* by LAMP assay even if these animals were positive in Kinyoun stain. This implies that the other oocysts detected by Kinyoun stain could be other *Cryptosporidium* species.

Cryptosporidium genotype 2 is probably the major source for environmental contamination. Xiao et al. [24] examined the species and strain-specific types of *Cryptosporidium* spp. in clinical and environmental samples. Results indicated that anthroponotic organisms (genotype 1) account for the majority of the cases and person-to-person transmission in non-outbreak cases. Meanwhile, genotype 2 largely causes human infection through contamination of water or food or direct contact with infected animals, especially in rural areas. Taken together, there are two distinct populations of *C. parvum* parasites: one cycling only in humans and one cycling predominantly in animals. The latter can sometimes cause human infections. Morgan et al. [25] reported that random amplified polymorphic DNA (RAPD) markers have revealed two distinct groups of human *C. parvum* isolates: one containing most human isolates and the other containing some human isolates and all animal isolates indicating the possibility of zoonotic infection.

In conclusion, the optimum condition for LAMP amplification of *C. parvum* DNA was 63°C for 60 min in heat block. This condition produced the desired visible results in fluorescent test, dye test and gel electrophoresis test. The ability of the LAMP product to emit a white glow under UV transilluminator was positive for fluorescent test. The ability of the LAMP product to show a green color after addition of SYBR dye was positive. In addition, the ability of the amplified DNA in the LAMP product to produce multiple ladder-like bands after gel electrophoresis indicated a positive result.

All fecal samples from cattle and water buffaloes were formerly detected to be positive for *Cryptosporidium* oocysts in Kinyoun stain. However, using LAMP assay, only 41% (7/17) were
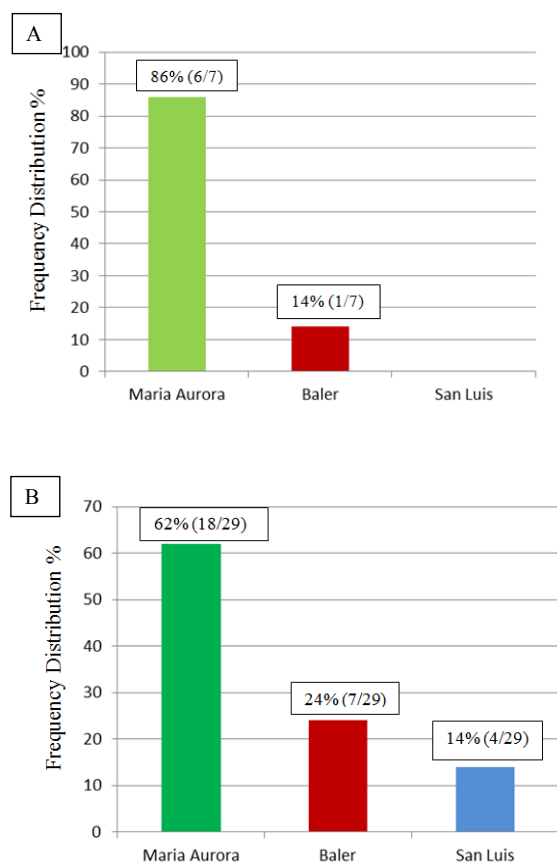
Fig. 4. Frequency distribution of *C. parvum* cases in A. cattle and B. water buffaloes using LAMP assay in the three municipalities of Aurora province

positive in cattle and 76% (29/38) in water buffaloes. Out of the seven LAMP positive cases in cattle, 86% (6/7) came from Maria and 14% (1/7) from Baler. Out of 29 LAMP positive cases in water buffaloes, 62% (18/29) came from Maria Aurora, 24% (7/29) from Baler, and 14% (4/29) from San Luis.

Comparing the results with the previous study [11], probable zoonoses of *C. parvum* between the farmers and their animal was determined. Eight farmers (positive in PCR) with their water buffaloes (positive in LAMP assay) were detected to have *C. parvum.* Only one farmer with his cattle was detected positive of *Cryptosporidium* spp. in PCR; however, it was negative in LAMP assay. Hence, non-*parvum* species infected the farmer and his cattle.

Acknowledgements

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