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Application of molecular tools in detecting zoonotic pathogens in organic fertilizers and liquid supplements

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ABSTRACT

The use of organic fertilizers and liquid supplements for crop production is rapidly growing as an alternative system to conventional agriculture. However, very little is known about the public health issues related to pathogens. This study endeavors to identify the important zoonotic pathogens with the current molecular diagnostic tools, Loop-mediated isothermal amplification (LAMP) and Recombinase polymerase amplification (RPA), against the conventional pathogen detection. These cost-effective molecular techniques have proven to be confirmatory tests of the target pathogens present in organic fertilizers and liquid supplements, which recommends an advancement for the comprehensive field surveillance-response approach in many developing countries with resource-limited settings quality assurance and safety implementation of organic biosolids for sustainable agricultural farming.

1. Introduction

It is common knowledge that the principal component of organic fertilizers is animal manure which has the presence of different kinds of microorganisms that can be infectious and zoonotic. Poor handling, sanitation, and processing of organic fertilizers and crops during production and harvesting can lead to various amounts of pathogens and even viruses running through the subsequent food chain. Currently, the Philippines lacks existing policies, guidelines or programs to control health issues concerning the use of organic fertilizers and liquid supplements. There is also a challenge on how local farmers can easily understand that organic biosolids can be contaminated with pathogens. There are limited studies in the Philippines conducted on the survey of pathogens in organic fertilizers. This gap accentuates the importance of awareness of the occurrence, extent, and impact of the microbial contamination concerns. Therefore, this study aims to evaluate recent molecular detection techniques and select the potential method for the rapid diagnosis of the essential pathogens that may be present in local organic fertilizer and liquid supplement, particularly the important zoonotic pathogens: Bacteria (*Salmonella* sp, *Escherichia coli*), protozoa (*Entamoeba histolytica*) and helminths (*Ascaris lumbricoides and Trichiuris trichiura*). Mainly, to investigate and compare the different molecular detection methods such as the polymerase chain reaction (PCR), the Loop-Mediated Isothermal Amplification (LAMP), and the Recombinase Polymerase Amplification (RPA) by positivity test rate in comparison with the conventional techniques in detecting these pathogens in organic fertilizers and liquid supplements.

The importance of molecular diagnostic methods for detecting soil-transmitted pathogens such as bacteria, protozoa, and helminths has emerged significantly over the years. The growing interest has occurred comparably with the belief that standard microscopy-based protocols for examining stool/soil samples are subpar, leading to misdiagnosis of infections. Furthermore, numbers of cysts, oocysts, eggs, and larvae excretion vary considerably within the collected samples, leading to false-negative results, primarily when non-amplification-based diagnostic methods are applied in the detection. Moreover, microscopy-based diagnostic methods have been associated with misidentification of pathogens due to morphological similarities for many species, resulting in a low-level infection undiagnosed and disease misdiagnosis [1]. In such matters, conventional and real-time Polymerase Chain Reaction (PCR)-based assays have been applied to improve both species-specificity and detection limits, which were demonstrated to be very useful and valuable in recent years. The molecular techniques make the identification and quantification of soil-borne pathogens more efficient and sensitive. With only small quantities of samples, defined gene sequences of soil-borne pathogens can be detected with PCR, quantitative PCR (qPCR), and other nucleic acid-based methods [2] such as loop-mediated isothermal amplification (LAMP) and Recombinase

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Polymerase Amplification (RPA). This study demonstrated that molecular detection could have advantages over conventional methodologies and improve early detection and management for the safe manufacturing of organic fertilizers and supplements.

2. Materials and methods

2.1. Sample collection

The samples were collected from the organic farms in Central and Northern Luzon in the Philippines. A total of 40 solid fertilizer (SF) and liquid supplement (LF) samples which consist of 14 standard organic fertilizers (S-OF), 4 vermicasts (VCA) solid fertilizer, 6 vermicompost (VCO), 1 compost grass (COG) solid fertilizer, 5 fermented fruit juice (FFJ) liquid supplement, 3 plant extract (PE) liquid supplement, 2 fish amino acid (FAA) liquid supplements, 2 commercial solid fertilizer (HP) and (NH), 1 commercial (IX) liquid supplement, 2 liquid supplement from organic farms in Nueva Ecija and La Union. Organic solid fertilizers were carefully transferred from a sterile ziplock bag while liquid fertilizers were poured into sterile 50 ml conical tubes. Samples were labeled carefully and transported back to the laboratory, and stored in the refrigerator before analyses.

2.2. Conventional method

2.2.1. Bacterial culture

Primary enrichment of samples was performed by mixing 1 g of solid fertilizer/1 ml liquid fertilizer into a sterile 50 ml conical tube with 10 ml buffered peptone broth and incubated at 37 °C for 18–24 h. After pre-enrichment, 100 μl was transferred into a 10 ml of Rappaport-Vassiliadis Soya Peptone (RVS) broth and incubated at 42 °C for 18–24 h. Eosin Methylene Blue Agar (EMBA), MacConkey Agar (MC), and Xylose Lysine Deoxycholate (XLD) plates were prepared in triplicates following the manufacturer's instruction (HiMedia, India). Ten loopful of RVS broth was streaked into plates with EMBA, MC, and XLD to detect *Salmonella* sp. and *E. coli,* respectively [3]. Samples with positive bacterial contamination were scored following the colony coloration: EMBA - metallic green sheen (*E. coli*.), MC – red/pink *(E. coli*), XLD – white (*E. coli*), and black *(Salmonella* sp.).

2.2.2. Microscopy

Examining samples for eggs and cysts was performed on the same day samples were collected to avoid shriveling or hatching. Each sample, in 3 replicates, was observed under the compound light microscope (400X) using acid-fast staining and Sheather's sugar flotation method to observed *E. histolytica* and *Ascaris lumbricoides*., respectively. Documentation was done using a digital camera, and the presence of eggs and cysts was scored accordingly using the Colmer-Hamood guide for fecal microscopy [4].

2.3. Molecular detections using PCR

2.3.1. Nucleic acid extraction

Total DNA molecules were extracted from the samples using i-genomic Stool DNA kit (iNTRON, Korea) following the manufacturer's protocol. In brief, SF and LF have weighed approximately 200 mg in a 1.5 ml spin column tube. Briefly, the nucleic acid extraction process was performed according to the manufacturer's protocol. The collected pellet was then appropriately washed, dried, and eluted with the buffer and quantified using IMPLEN P330 Nanophotometer (Denmark) to determine the concentration (ng/μL) and purity at A260/A280 nm (data not shown).

2.3.2. Optimization of primers and PCR-based detection

Positive control for each pathogen was obtained accordingly to optimized the primers designed for amplification. In brief, purified DNA of *Salmonella* sp. and *E. coli* were obtained from SF vermicast from a backyard farm in Bulacan and an organic farm in Nueva Ecija, respectively. On the other hand, Pure DNA of *E. histolytica* was acquired from the Natural Sciences of Research Institute (NSRI), University of the Philippines, Diliman. *A. lumbricoides* DNA was obtained from a seven-year-old child in Bulacan. The sets of primers for each pathogen were screened using BioRad T100 Thermal Cycler (Bio-Rad, USA) with their corresponding annealing temperatures and cycling profile. The 10-μl PCR reaction mixture contained 1 μl of template DNA, 0.1 μl Taq DNA Polymerase, 1.5 μl MgCl2, 1 μl dNTPs, 2 μl 5X PCR Buffer, 2.4 μl nuclease-free water (NFH2O) and 1 μl each of forward and reverse primers. The PCR cycling conditions were as follows: 95 °C for 10 min, ran for 39 cycles at 94 °C for 1 min, 50 °C for 30 s and 72 °C for 1 min, with a final elongation at 72 °C for 7 min. PCR products were analyzed in a 1.5% agarose gel stained with GelSafe Red dye (iNTRON, Korea, and viewed under UV light to score positive results.

2.3.3. Loop-mediated isothermal amplification (LAMP) assay

The obtained sequences from the positive controls were used to design 3-sets of LAMP primers: F3, B3, Forward-Inner Primers (FIP), Backward-Inner Primer (BIP) using Primer Explorer V. 5.0 (Japan). The 12.5 μl LAMP reaction mixture contained, 2.0 units Bst Polymerase, 1.2 M Betaine, 0.8 μM dNTPs, 2 μl 10X Buffer B, 0.2 μM F3 and B3 primers each, 6 μM FIP and BIP primers and the DNA template. Optimization of LAMP primers set was carried out with gradient temperature ranging from 60 °C – 65 °C at incubation time of 30 min–90 min and terminated at 85 °C for 2 min. LAMP assay results were validated by qualitative assessment of color changes from orange to yellow through the addition of 1.0 μl 10X SYBR Green Dye (Thermo Fisher Scientific, USA). For confirmation, LAMP products were then loaded in a 1.5% agarose gel stained with GelSafe Red dye (iNTRON, Korea), electrophoresed at 100 V for at least 45 min s, and viewed under UV light to score ladder-like bands indicating a positive result.

3. Results and discussion

The conventional method of detection. Conventional detection through bacterial culture revealed that 19% and 37% of samples are positive of Salmonella spp. and *E. coli*, respectively (Fig. 1). Also, microscopic examination shows suspected E. histolytica cysts (3%) in the samples, including other parasites such as hookworm egg, hookworm larvae, unidentified protozoa, and bacteria. *Toxoplasma gondii* cyst was also suspected in microscopic analysis (Fig. 2). Other nematodes were found in hookworm eggs and T. gondii oocyst, as shown in Fig. 2E and F, respectively. Nine samples were recorded to have bacterial colonies of *Salmonella* (Fig. 2A). On the other hand, *E. coli* is predominantly observed in the samples, as confirmed by its metallic green sheen in EMBA plates and dark pink colonies in MC (Fig. 2B and C). Other colonies not belonging to *Salmonella* and *E. coli* were also observed from the cultures but not included in the current study. The colonies from the cultures were individually inoculated and were subjected to further enrichment for PCR-based confirmation. Results confirmed that the obtained colonies from the bacterial culture belonged to *Salmonella* sp. and *E. coli* (data not shown).

PCR, LAMP, and RPA targeting the specific genes demonstrated to have an advantage in detecting these pathogens. From all the samples assayed, PCR detected *Salmonella* sp., 44%; *E. coli*, 56%; *E. histolytica* 11%; *C.* parvum, 37%; *A. lumbricoides,* 26% and *T. trichiura,* 30%. LAMP presented the positivity rates of *Salmonella* sp., 67%; *E. coli*, 63%; *E. histolytica,* 44%; *C. parvum,* 52%; *A. lumbricoides,* 41%, and, *T. trichiura,* 44%. RPA technique was applied with the same molecular markers with LAMP. Correspondingly, RPA obtained the following positivity rates: *Salmonella* sp*., 48%; E. coli, 56%; E. histolytica,* 37%; *C. parvum,* 44% *A. lumbricoides*, 37% and *T. trichiura,* 41% (Fig. 1). The results imply that identifying bacterial pathogens using culture media can be uncertain, given that some bacterial cells may not be taken up

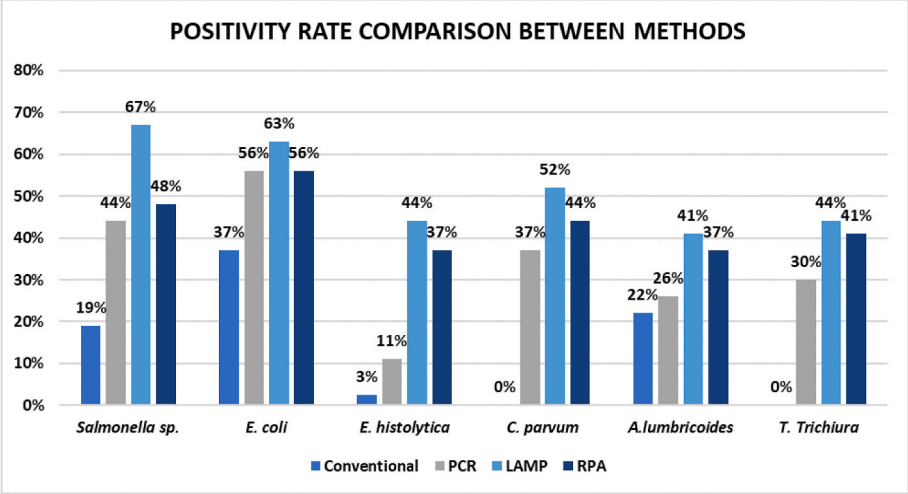

Fig. 1. A. Comparison of conventional and molecular-based detection methods of pathogens. Positivity rate results of PCR-, LAMP-, and RPA-based detection reveal that scores of organic fertilizers are contaminated with pathogens that were not detected with the conventional methods. The overall positivity rate shows that LAMP has the highest rate.

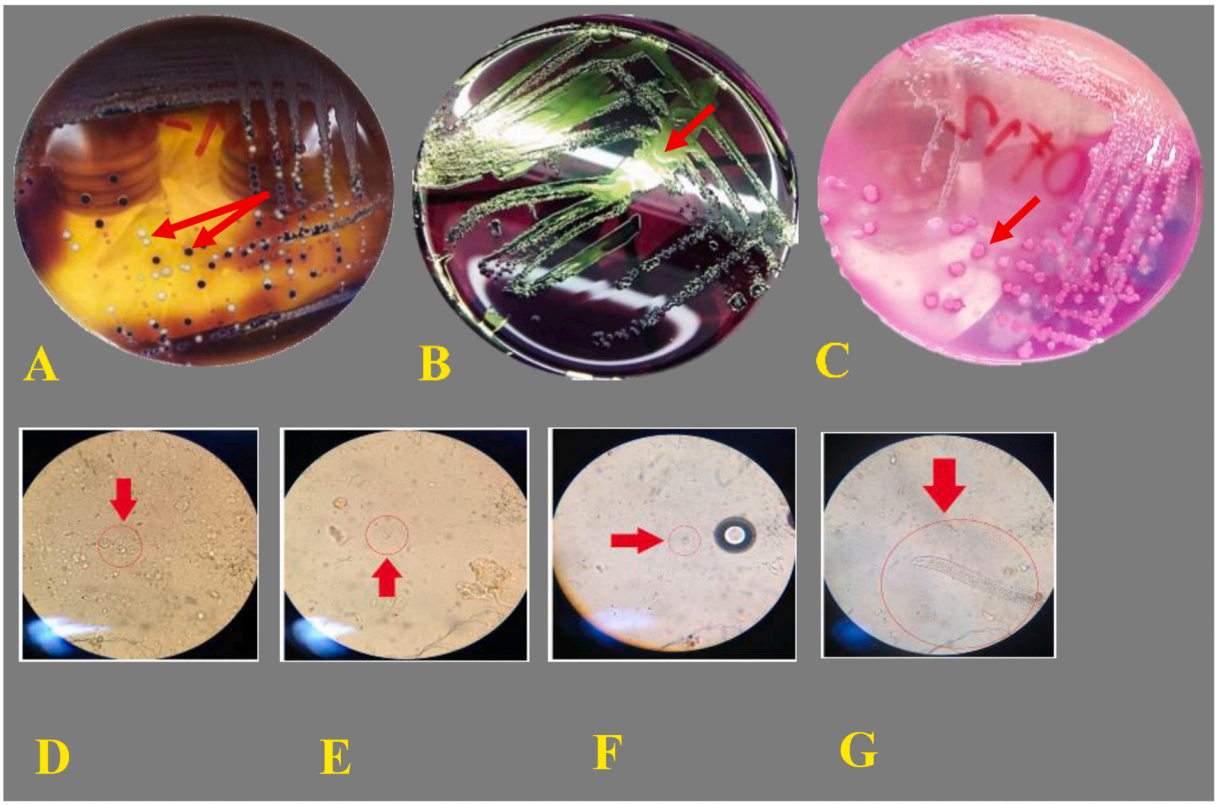

Fig. 2. Representative positive samples scored using the conventional method of pathogen detection. A.) XLD with *Salmonella* sp. with black centered colonies and *E. coli* with white colonies (arrow) B.) The presence of *E. coli* was further confirmed in EMBA, showing metallic green sheen (arrow). C.) Red-pink colonies (arrow) were also observed in MC, indicating the presence of *E. coli*. D.) *E. histolytica* cyst (red arrow) were observed in some of the samples. E.) Other pathogenic agents such as hookworm eggs indicating the diversity of pathogenic contamination of biosolids. F.) *Toxoplasma gondii* oocyst recorded from few samples. Hookworm larvae G.) Total Magnification: 400×. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

during the culture. Furthermore, several samples were confirmed positive with *E. histolytic*a and *A. lumbricoides* in molecular methods but were not identified in the conventional methods. Previous undetected microscopy examination was confirmed positive with the molecular techniques. Similarly, *T. trichiura,* where no eggs were found during microscopy examination, were confirmed positive using PCR (Fig. 3). LAMP shows higher positivity rates among the techniques tested (Fig. 1), which was confirmed by several other reports. Fig. 3 shows representative results from the samples tested, indicating the validity of PCR contrasted with the conventional methods.

LAMP assay can also be used to determine the presence of pathogens in organic fertilizers. Samples assayed for LAMP for *Salmonella* sp. and *E. coli E. Histolytica, C. parvum, A. lumbricoides* were successfully optimized for LAMP assay as shown in Fig. 4. A set of LAMP primers were

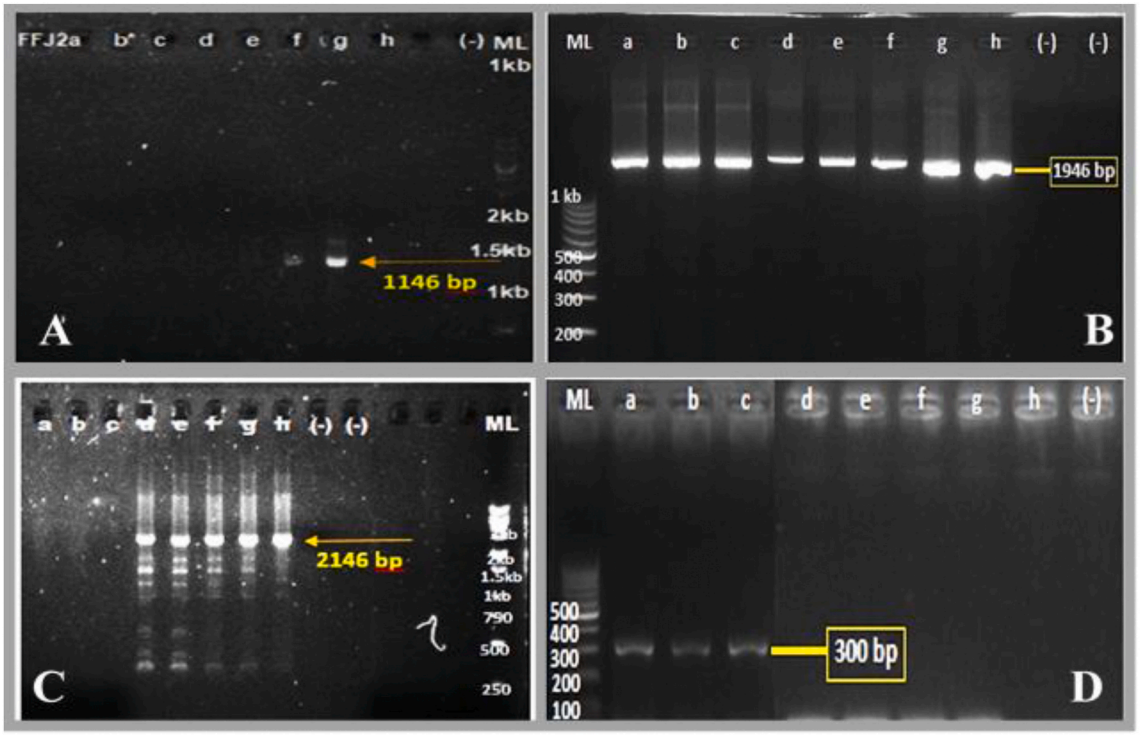

Fig. 3. Gel electrophoresis confirmed the amplification of the molecular target marker of each pathogen after PCR assay. Molecular Ladder (ML) – 1 kb, (a–h) genomic extracts from the samples, (−) Negative control. A.) glxk gene (1146 bp) of *E. coli*. B.) ssRNA gene (1946 bp) of *E. histolytica*. C.) InvA gene (2146 bp) of *Salmonella* sp. D.) ITS-1 gene (around 300 bp) of *A. lumbricoides*.

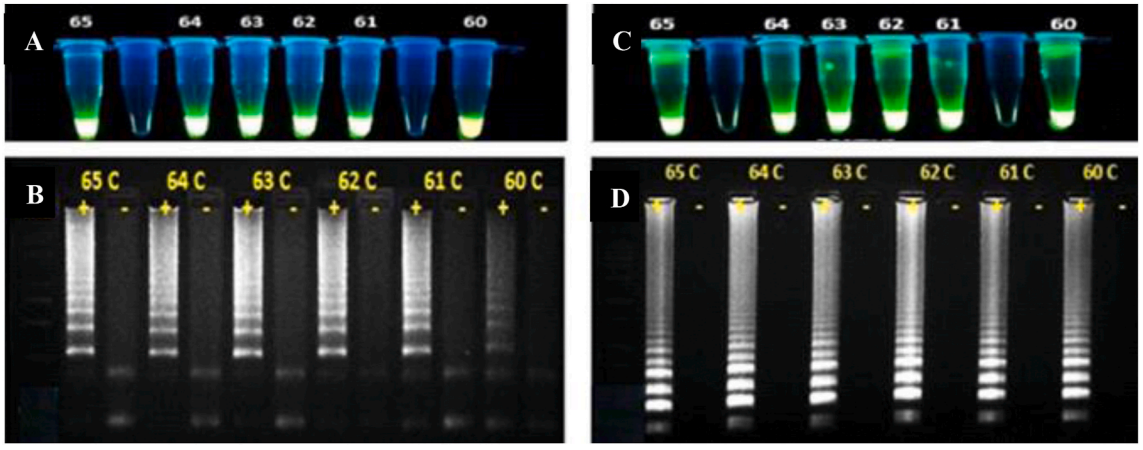

Fig. 4. LAMP assay for *Salmonella* sp. (A–B) and *E. coli*. (C–D). Addition of SYBR Green dye for visualization under UV light or blue LED light for positive amplifications (A and C). Positive results indicated a fluorescence. Gel electrophoresis of positive samples shows ladder-like bands due to different amplification sizes derived from the inner and outer markers. 65 °C – 60 °C (gradient isothermal temperatures for 1hr), + (positive control), - (negative control). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

tested and optimized at an isothermal temperature between 60 °C to 65 °C and amplified for 1 h.

Recombinase polymerase amplification (RPA) assay can also be combined with LAMP in detecting pathogens in organic fertilizers and liquid supplements. RPA was reported to be a highly sensitive isothermal amplification technique, operating at 37–42 °C, with minimal sample preparation and capable of amplifying as low as 1–10 DNA target copies in less than 20 min. The manner of demonstrating the results of RPA is the same with the LAMP applying similar primer sets. Positive reactions exhibit green fluorescence, and negative reactions appear light to whitish orange. Representative reactions are demonstrated.

4. Summary and conclusion

Zoonotic pathogens have caused severe food and disease outbreaks linked to a high microbial quantity of raw agricultural products [5]. Contamination of food crops is possible whereby these pathogens could pose a great risk to human health if present in significant amounts. Pathogens of concern that may be present include: Bacteria (*Salmonella* sp. *Escherichia coli*), protozoa (*Cryptosporidium parvum.*, *Entamoeba histolytica*, *Toxoplasma gondii*), and helminths (*Ascaris lumbricoides*, *Trichuris trichiura*) [6–8]. Some research on the contamination of crops reported that microbial contamination is caused by several causes such as exposure to microbiologically loaded lands, poor drainage system,

animal feces used as organic fertilizers, lack of sanitation, and workers' proper hygiene during pre-and postharvest [9,10,11and12]. Moreover, when it comes to the allowable level of pathogens, *Salmonella* must be non-detectable, and fecal coliform levels must not exceed $<5 \times 102$ CFU/g of dry weight in biosolids [13]. Initial results show that *E. coli* and *Salmonella* were primarily observed in the samples. *E. coli* is commonly found in the lower intestine of mammalian species [14]. Most *E. coli* do not cause infections to humans as they are part of the gut's normal microflora. However, virulent strains can cause gastroenteritis, urinary tract infections, neonatal meningitis, hemorrhagic colitis, and Crohn's disease through fecal-oral transmission.

Bacterial growth of *Salmonella* sp. and *E. coli, E. histolytica, A. lumbricoides, T. gondii, C. parvum, and T. trichiura* were processed for conventional and molecular methods. Using the specific primers indicated. Samples positive on bacterial culture also turned out positive on PCR, LAMP, and RPA assays. The application of LAMP and RPA assays on the molecular diagnostic of microorganisms offers a new technique to obtain fast and reliable results. According to this study, LAMP shows a higher positivity rate among the techniques tested.

Several studies have already used LAMP assay to detect pathogenic agents in agricultural sectors [15,16]. Another molecular tool named RPA is also good detection of pathogens along with the LAMP technique. RPA offers a great potential for lower cost, higher speed since no equipment is utilized. Isothermal amplification techniques can become superior to standard PCR for cost-efficiency that benefit countries with limited resources. However, the real-time PCR possesses advantages beyond that of the conventional methods, such as increased sensitivity, but the technique has several limitations. The instrument is itself, and the consumables are costly as compared with the conventional methods. The technical and standardized protocols are limited and prone to inhibitors, reagents can be costly and advanced expertise and technical skills are required for performing PCR assay. It is also restricted to laboratories with good financial support.

On the other hand, the isothermal amplification techniques such as LAMP and RPA can be field-based without requiring thermal cyclers, and monitoring can be done with visual, naked-eye observation. It requires shorter steps than PCR and is exceptionally resilient to inhibitors, non-target DNA, physical and chemical parameter modifications, reagents exposure to ambient temperature, and prolonged-time preparing the reactions. Therefore, crude extract can be utilized. Equally important is developing a fast, simple, low-cost yet reliable, sensitive, and specific nucleic acid-based diagnostic technique that would serve as a screening protocol detecting the zoonotic pathogens in organic fertilizers and liquid supplements would benefit the farmers in developing countries.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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