Development of nested PCR for identification of *Entamoeba coli* in stool samples

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Research

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Abstract

Background

The accurate detection of *Entamoeba* species provides for a better understanding of the nature of these parasites in humans and helps to provide accurate epidemiological data. However, the microscopic method is not sensitive and specific to identify the precise identification of *Entamoeba* spp. in stool specimens especially if it was examined by inexperienced laboratory technicians. In this study, nested PCR was developed for detection of *Entamoeba coli*, and can be differentiated from the other human *Entamoeba* spp. especially *Entamoeba histolytica, Entamoeba dispar*, and *Entamoeba moshkovskii*.

Methods

Nested PCR assay was performed using *Entamoeba* genus-specific primers for the first round PCR reaction and newly designed *E. coli*-specific primers targeting small subunit ribosomal RNA (SSU rRNA) gene in the second round. Both sensitivity and specificity of nested PCR for detection of *E. coli* were determined. This assay was validated for detection of *E. coli* by using DNA obtained from 55 stool samples which previously examined by microscopy. Results obtained from developed nested PCR, genus-specific PCR, and microscopy were analyzed.

Results

Nested PCR was successfully developed to diagnose *E. coli* infection with a product size of 166 base pairs (bp). The nested PCR method could detect as little as 1 pg of *E. coli* DNA. The high specificity was confirmed by testing with DNA from the different intestinal pathogens, including bacteria and other protozoa. After testing with all stool samples, 50.9% of tested samples were positive for *Entamoeba* spp. by genus-specific PCR assay, 32.7% of *E. coli* positive samples detected by nested PCR, and 29.1% were positive by microscopic examination. Interestingly, four samples previously negative for *E. coli* by microscopy demonstrated *E. coli* by nested PCR. In addition, microscopy showed two false-positive compared with both nested PCR and genus-specific primers results.

Conclusions

The developed nested PCR assay based on genus *Entamoeba*-specific primers and newly designed *E. coli*-specific primers shows high sensitivity and specificity for the diagnosis of *E. coli* in stool samples. This nested PCR assay will be beneficial not only for diagnostic purposes but also for a better understanding of the epidemiology of *E. coli* in the human population worldwide.

Background

In the genus *Entamoeba*, there are many reported species, some of which are known to infect humans. So far, nine species of the genus Entamoeba are found in humans which are *Entamoeba histolytica*, *Entamoeba dispar*, *Entamoeba moshkovskii*, *Entamoeba bangladeshi*, *Entamoeba coli*, *Entamoeba polecki*, *Entamoeba hartmanni*, *E. gingivalis*, and *E. chattoni* [1–3]. Approximately, 500 million people could be infected with *Entamoeba* worldwide; only 10% of them have been infected with *E. histolytica* and the rest other nonpathogenic species [4] and *E. histolytica* caused millions of cases of amebiasis and over 50,000 patients were dead annually [5]. *E. coli* is one of the non-pathogenic protozoa and most commonly found in the human intestinal tract [6, 7]. *E. coli* is distributed globally and the highest prevalence was reported in rural areas and regions with poor sanitation [8]. Recently, the high prevalence of 25.7–35% was reported in some rural school communities in Colombia [9, 10]. The transmission is the fecal-oral route and it is usually considered as a non-pathogenic organism [11]. Interestingly, *E. coli* was able to phagocytose red blood cells with different efficiency among strains but this ingestion may be rarely seen in stool samples [12, 13]. However, *E. coli* has been reported that it might be involved in cases of diarrhea and gall bladder disease [14–17]. In cases of heavy infection of *E. coli*, patients may complain of indigestion, gastritis, dyspepsia however symptoms including loose stools, flatulence, and colicky abdominal pain were rarely shown in patients [15], and the patient infected with *E. coli* has gastrointestinal complaints [1]. In addition, *E. coli* was reported as an indicator of poor nutrition status in school children [18].

Normally, human *Entamoeba* spp. can be diagnosed by using microscopy and mainly based on the size and number of the nucleus. Some difficulties have been found when the microscopic examination was used for parasite detection by inexperienced laboratory technicians. Moreover, there are overlapping of their morphologic features in some circumstances that lead to misdiagnosis especially *E. coli* from *E. histolytica*, *E. dispar*, *E. moshkovskii*, and *E. bangladeshi* which showing the overlapping in their size range. The higher sensitivity and specificity methods were needed and developed [19, 20]. In addition, reports of all *Entamoeba* spp. in fecal samples are essential to understand their epidemiology in particular areas. Although, several molecular methods have been established to diagnose *E. histolytica*, and even more some of them were able to differentiate *E. histolytica* from *E. dispar* and *E. moshkovskii* [21–26], a few methods have been developed to detect *E. coli* from stool samples. In 2003, a reverse line hybridization assay was used to detect *E. coli* [27]. After that, a combination of PCR and DNA sequencing could identify *E. coli* from human stool samples [28], and PCR using specific primers was able to detect *E. coli* in captive Japanese macaques and chimpanzees stool samples [29, 30].

In this study, the development of nested PCR targeting SSU rRNA gene of *E. coli* was performed for detection of *E. coli* in stool samples, and its sensitivity and specificity were determined. The development of the high sensitivity and specificity for the diagnosis *of E. coli* in clinical specimens will provide a more accurate diagnostic result for human *Entamoeba* infections and will be beneficial in epidemiological studies of *E. coli* in the future.

Methods

Fecal samples and microscopic examination

A total of 55 DNA samples extracted from stools which previously examined by microscopy were used to validate the nested PCR assay developed in this study (Additional file 1: Table S1). Twenty-nine DNA samples were from protozoa microscopy positive stools, and 19 out of them were from *Entamoeba* spp. positive samples; *E. histolytica* (n=1), *E. coli* (n=16), and *E. hartmanni* (n=2) (Table 1). *E. coli* (07-286 strain) DNA kindly provided by Dr. Charles Graham Clark (London School of Hygiene and Tropical Medicine, London, United Kingdom) was used as a positive control. This study has been approved by the Ethics Committee, Faculty of Tropical Medicine, Mahidol University, Bangkok (TMEC 19-002).

Table 1
Microscopic examination of parasitic protozoa from 55 stool samples.

Protozoa	Number of positive samples (%)
Negative	26 (47.3)
Positive	29 (52.7)
Entamoeba histolytica	1 (1.8)
Entamoeba coli	16 (29.1)
Entamoeba hartmanni	2 (3.6)
Endolimax nana	13 (23.6)
Blastocystis hominis	13 (23.6)
Giardia duodenalis	4 (7.3)
The protozoa found in mixed infections are presented as separate detections.	

Construction of E. coli nested PCR primers

Entamoeba coli specific primers were designed based on small-subunit rRNA gene sequences (accession no. AF149915) from GenBank using Primer 3 software. The nucleotides sequences were aligned using the Multialin program (http://bioinfo.genopole-toulouse.prd.fr/multalin/multalin.html). All primers were blasted to sequences in GenBank for homology.

Detection of Entamoeba spp. using genus-specific primers

DNA of fecal samples was extracted by using a QIAamp stool DNA extraction kit (QIAGEN, Hilden, Germany). The extracted DNA was then stored at 20°C until use. Genus-specific PCR amplification was performed using previously designed primers [31] in a final volume of 30 µl containing 1X PCR buffer, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 25 pmol of each genus-specific primer, 1 unit of *Taq* DNA polymerase (Amersham) and 2 µl of DNA samples. Reactions were carried out on *Px2* Thermal Cycler (ThermoHybaid, United Kingdom). Samples were denatured at 94°C for 5 min and then subjected to 35 cycles of 94°C for

1 min, 55°C for 1 min and 72°C for 1 min, following by a final extension at 72°C for 7 min. PCR products obtained by using genus-specific PCR primers, were analyzed and identified by electrophoresis in 1.5 % agarose gels (TBE buffer). The expected size of the genus *Entamoeba* PCR product was 550 bp.

Nested PCR assay for detection of E. coli **from fecal samples**

A nested PCR assay was developed for the detection of *E. coli* from stool samples. For the first round PCR reaction, all DNA samples were examined for genus *Entamoeba* by using genus-specific primers as described above. In the second round, the amplification of *E. coli* specific DNA fragment by touch-down condition was applied. Amplification of target DNA in each reaction mixture contained 50 µM each dNTP, 0.5 µM of each forward and reverse primers, 1.5 mM $MgCl_2$, 0.5 units of *Taq* polymerase, 1x PCR buffer, and 1 µl of PCR product amplified by using genus-specific primers. The PCR assay was carried out with initial denaturation at 94^{o}C for 5 min, followed by 25 cycles of touch-down PCR consisting of denaturation at 94^{o}C for 30 sec, the primer annealing from 68^{o}C to 60^{o}C for 30 sec and extension at 72^{o}C for 30 sec. To increase the product yield, the touch-down PCR step was followed by 10 cycles of 94^{o}C for 30 sec, primer annealing at 60^{o}C for 30 sec, and extension at 72^{o}C for 30 sec, and finally one cycle of extension at 72^{o}C for 2 min. The amplified products were run on 2.0% agarose gels using TBE buffer followed by staining with ethidium bromide and visualized on a UV transilluminator. The nested PCR product of *E. coli* positive sample should show an expected size of 166 bp on an agarose gel.

Determination of the sensitivity and specificity of nested PCR assay

The sensitivity of nested PCR was determined by ten-fold serial dilutions of *E. coli* genomic DNA using the protocols described above. The final amount of DNA in the serial dilutions were 100, 10, 1, and 0.1 pg respectively. The specificity of nested PCR assay was tested against several DNAs from other intestinal protozoa and bacteria including *Blastocystis hominis, Cryptosporidium spp., Giardia duodenalis*, *E. histolytica, E. dispar*, *E. moshkovskii*, *Escherichia coli, Salmonella* sp., and *Shigella* sp. DNA extracted from a parasite-free stool sample was used as a negative control.

Results

Microscopic examination

Based on microscopic examination, 29 out of 55 stool samples (52.7%) were positive for protozoan infection (Table 1). Of these examined samples, 34.5% (19/55) were suspected to contain *Entamoeba* spp. Most of the *Entamoeba* spp. were identified as *E. coli* (29.1%), while *E. hartmanni* (3.6%) and *E. histolytica* (1.8%) were found. In addition, the other well-known pathogenic protozoa, *Giardia intestinalis* was identified in 7.3% of all tested samples whereas *E. nana* and *B. hominis* were equally detected at 23.6% (Table 1). The intestinal parasites found in all samples were either single or mixed infections. The

mixed infection was 21.3% (16/55) of all tested samples; it was as high as 55.2% (16/29) of the protozoa-positive samples (Additional file 1: Table S1).

Construction of E. coli nested PCR primers

All primers were constructed based on *E. coli* SSU rRNA gene sequences; accession no. AF149915. This sequence database was obtained from the National Centre for Biotechnology Information (NCBI). The nucleotide sequences of primers were successfully designed for *E. coli* nested PCR assays were shown in Table 2. All constructed primers were blasted to GenBank for homology study; the results showed that all primer sequences were homology to *E. coli* therefore suitable for species identification. The *E. coli* specific PCR primers generate 166 bp of amplicon which was corresponding to the expected product for *E. coli* DNA.

Table 2
Oligonucleotides sequences of *E. coli* specific PCR primers constructed from small sub-unit rRNA gene (Gen Bank Accession No. AF149915).

Primers code	Nucleotide sequence 5' to 3'	Tm (^{0}C)
EcoliF (forward primer)	5'- CTA AGC ACA AAG TCC TAG TAT GAT G - 3'	61.3
EcoliR (reverse primer)	5'- CCT CAT CGA TTA CAC TCC CAG AG -3'	64.6

Detection of genus Entamoeba from stool samples by PCR

According to specific primers of genus-specific PCR assay for *Entamoeba* were used, DNA of all *Entamoeba* species should be detected. The *Entamoeba* genus-specific PCR primers generated 550 bp amplicon in positive samples as shown in Fig. 1. Amplification products showed that 50.9% (28/55) was positive for *Entamoeba* spp. while 49.1% (27/55) was negative (Table 3). Positive cases of *Entamoeba* spp. were found in double compared with microscopic examination. All samples were further examined for *E. coli* by nested PCR.

Table 3
Results of microscopic examination and the nested PCR for detection of *E. coli* in stool samples (n = 55) compared with genus *Entamoeba*-specific PCR assay.

Results	Microscopy Number (%)	Nested PCR Number (%)	Genus-specific PCR Number (%)
Positive	16 (29.1)	18 (32.7)	28 (50.9)
Negative	39 (70.9)	37 (67.3)	27 (49.1)

Detection of E. coli by nested PCR assay

A nested PCR assay for the detection of *E. coli* was successfully developed. By using the designed primers of *E. coli*, nested PCR amplification produced a fragment of 166 bp corresponding to the expected products from *E. coli* (Fig. 2). The sensitivity of this assay was evaluated using series of ten-fold serially diluted of *E. coli* genomic DNA, and results demonstrated that the assay was able to detect as little as 1 pg for *E. coli* DNA (Fig. 3). The minimum detection level of the assay suggests that it is sensitive enough for the accurate identification of *E. coli* in stool specimens. Based on results, there was no cross-amplification observed when the assay was tested against parasite-free stool DNA sample or against any genomic or infected stool DNA containing different bacterial or protozoal pathogens (Fig. 4).

Evaluation of the nested PCR assay in stool samples

DNA of 55 stool samples was used to evaluate the nested PCR assay. Some samples were reported as positive for *Entamoeba* cysts by microscopic examination or the other intestinal protozoa. After stool examination using the nested PCR assay with *E. coli* specific primers, 166 bp fragments shown in Fig. 5 were found in 18 samples (32.7%), indicated that they were positive for *E. coli* whereas 37 of them (67.3%) were negative. Nested PCR could detect four E. positive samples which were not detected by microscopy. Moreover, two false-positive samples were reported by microscopic examination while they were negative by both nested PCR and genus-specific PCR assays (Additional file 1: Table S1).

Discussion

Entamoeba spp. in stool samples is normally identified by using traditional microscopic examination based on morphological features and this method has been used for diagnosis of amebiasis in a routine laboratory until now. However, light microscopy cannot be able to differentiate all *Entamoeba* spp. except identification them at the genus level. In addition, overlapping of their morphologic features might be found depending on the circumstances. Thus, molecular methods will be helpful to clarify the identification of *Entamoeba* spp. In this study, the nested PCR assay using genus-specific primers and the specific primers designed for *E. coli* was successfully developed. *E. coli* comprises two major clades that have been named ST1 and ST2, and ST1 was related to humans while ST2 was found mostly in non-human hosts [6]. Based on sequence divergence, it was suggested to consider *E. coli* ST1 and ST2 to be distinct species; however, there are no other known differences between the two subtypes so far [6, 32]. In the present study, *E. coli* primers were therefore designed according to *E. coli* SSU rRNA gene sequences; accession no. AF149915 which was classified as *E. coli* ST1 for detection of *E. coli* in stool samples whereas *E. coli* ST2 is represented by SSU rRNA gene sequences; accession numbers AF149914 and AB444953 and suitable for detection of *E. coli* in non-human hosts [6].

Results showed that the developed nested PCR assay is sensitive, detecting as little as 1 pg of DNA for *E. coli* with a dense product band (Fig. 3). Although there were a few reports using PCR to detect *E. coli* in humans, macaques and chimpanzees stool samples [28–30], the sensitivity of these assays was not shown. Therefore, we are unable to fully compare the relative sensitivity of the developed PCR assay. In

comparison with the PCR assays for differential detection, *E. histolytica*, *E. moshkovskii* and *E. dispar*, a limit of detection obtained from *E. coli* nested PCR (1 pg) is 10-fold lower than both *E. histolytica* and *E. moshovskii* (10 pg), and 20-fold lower than *E. dispar* (20 pg) [21]. According to the results, the sensitivity of this nested PCR assay is sufficient for the identification of *E. coli* in stool specimens. Since, none amplified PCR products were detected when DNAs from *E. histolytica*, *E. dispar*, *E. moshkovskii*, and other intestinal protozoa, bacteria, or humans, therefore, the high specificity of the assay is clearly shown (Fig. 4). *E. coli* was mostly found in protozoan infection in the tested samples using both microscopy (Table 1) and nested PCR assay (Table 3). Our finding is correlated to the previous study reporting that *E. coli* is more commonly found in stool samples than the other *Entamoeba spp*. [1]. In comparison to microscopic examination, a nested PCR assay could detect two more *E. coli* positive infections (Table 3). In addition, two negative samples of protozoan infections examined by microscopy were positive for *E. coli* by both nested PCR and genus-specific PCR. Moreover, microscopy could detect only 57% (16/28) of *Entamoeba* spp. positive samples compared with genus-specific PCR (Table 3). Based on the result, it indicates the difficulty faced by technicians to detect and differentiate the cysts of specific species of protozoa using microscopy for routine diagnosis. This finding confirms that the microscopic method may not be sensitive and specific to identify the precise identification of *Entamoeba* spp. in fecal specimens [33, 34]. The 11 samples negative for *E. coli* by our nested PCR assay should belong to other Entamoeba species, as shown by a positive Entamoeba genus-specific PCR result (Table 3).

Apart from the human host, *E. coli* was found in the top four of *Entamoeba* species, following *E. dispar*, *E. chattoni*, *E. histolytica* in experimental nonhuman primates [35]. Moreover, the contamination of raw vegetables and fruits with *Entamoeba* spp. cysts was globally documented [36] and *E. coli*, *E. histolytica* and *E. dispar* were the most commonly detected species in the collected samples [36–40]. Undoubtedly, it will be useful to include the *E. coli* set of primers in any epidemiological studies, and all species of *Entamoeba* should be reported. The true prevalence of individual species will help us to understand its global distribution and prevent unnecessary treatment in non-pathogenic species of human amoeba.

Conclusions

The new specific primers for *E. coli* were designed and the nested PCR assay was developed for detection *E. coli* in human stool samples from Thailand. We have demonstrated that this nested PCR assay is highly sensitive and specific for the identification of *E. coli* in stool samples. This nested PCR assay can be applied as an alternative tool for accurate diagnosis and in epidemiological studies of *E. coli*. This method will provide better epidemiological data and a greater understanding of infections with this amoeba in humans.

Abbreviations

SSU rRNA: small subunit ribosomal RNA; PCR: polymerase chain reaction; TBE: Tris-borate-EDTA

Declarations

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Authors' contributions

PR and PCP conceived and designed the experiments. PR and CN performed experiments and data analysis. KT participated in collecting microscopic examination data. PR prepared the manuscripts and artworks. PCP performed data analysis, prepared and edited the manuscript. All authors have read and approved the submitted manuscript.

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Availability of data and materials

Data supporting the results and conclusions of this article are included within the published article.

Ethics approval and consent to participate

In this study, stored stool samples kept in the Department of Protozoology, Faculty of Tropical Medicine, Mahidol University were used. This study was approved by the Ethics Committee, Faculty of Tropical Medicine, Mahidol University, Bangkok (TMEC 19-002).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

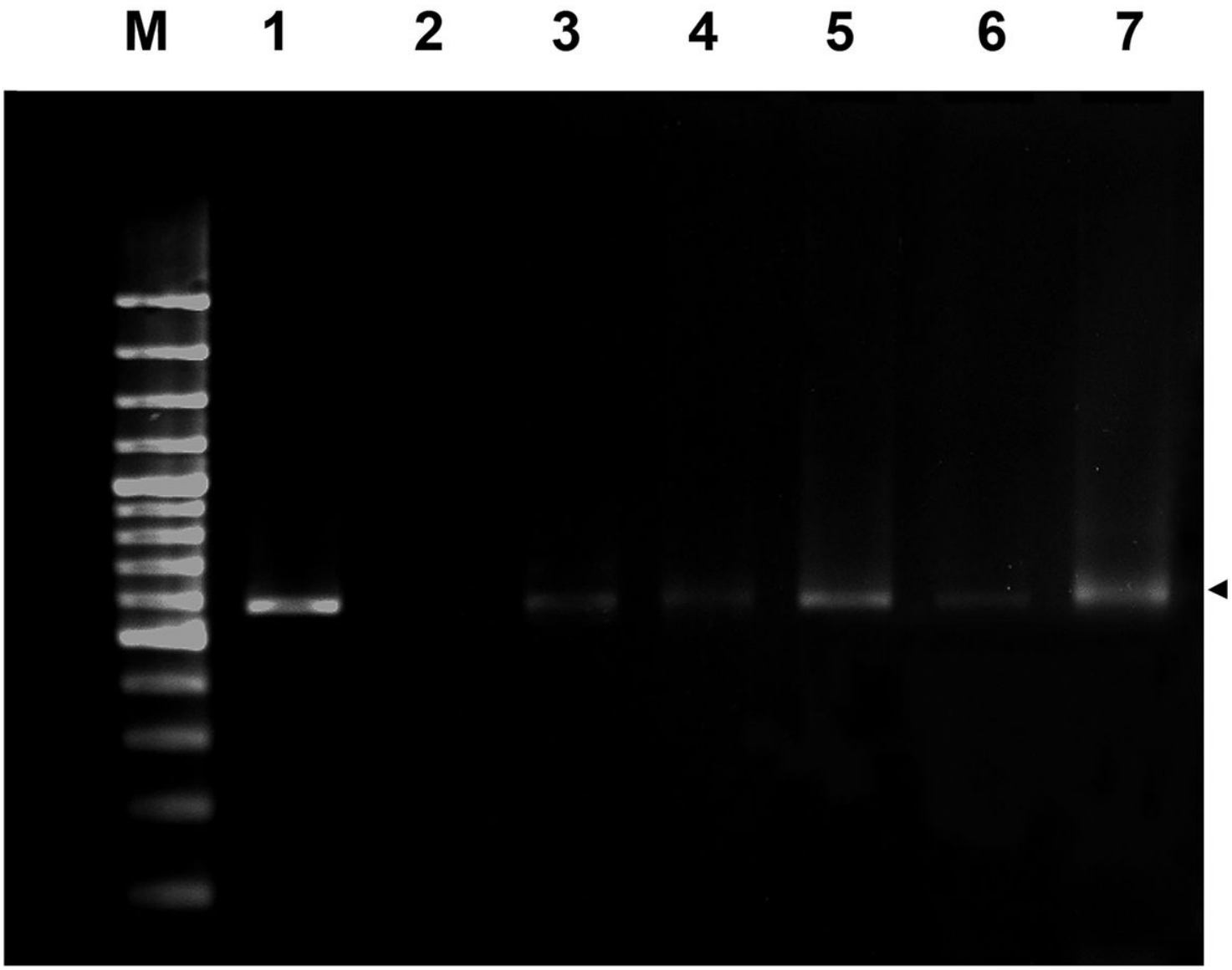

Figure 1

PCR amplification for detection of Entamoeba species using genus-specific primers. Lane M, 100-bp ladder DNA marker; lane 1, E. coli DNA as a positive control; lane 2, a reaction without DNA as a negative control; lanes 3 to 7, amplification products of positive samples for Entamoeba spp.(550 bp).

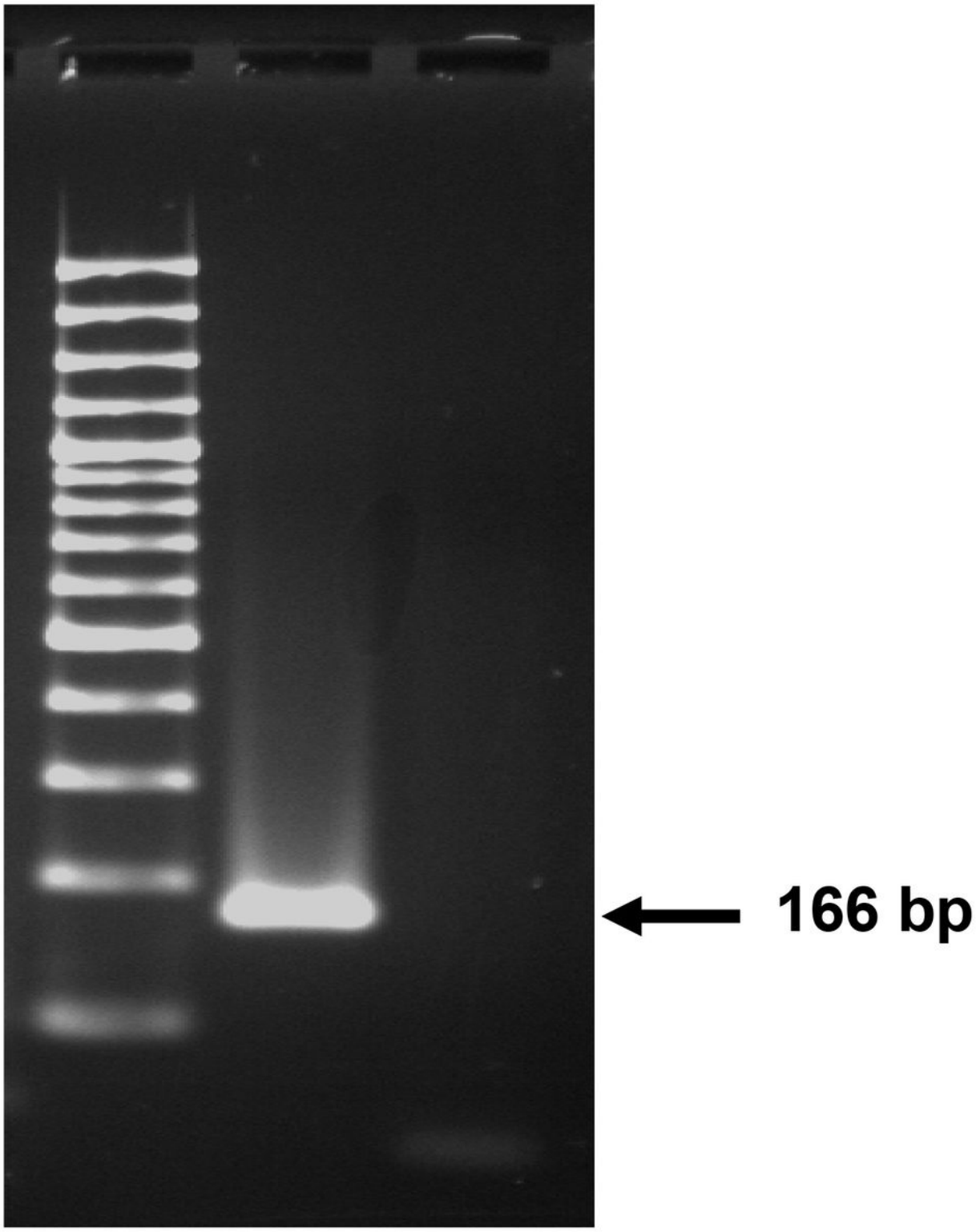

Figure 2

Gel electrophoresis of the amplification product of E. coli DNA by using the nested PCR. Lane M, 100-bp ladder DNA marker; lane 1, E. coli DNA; lane 2, a reaction without DNA as a negative control.

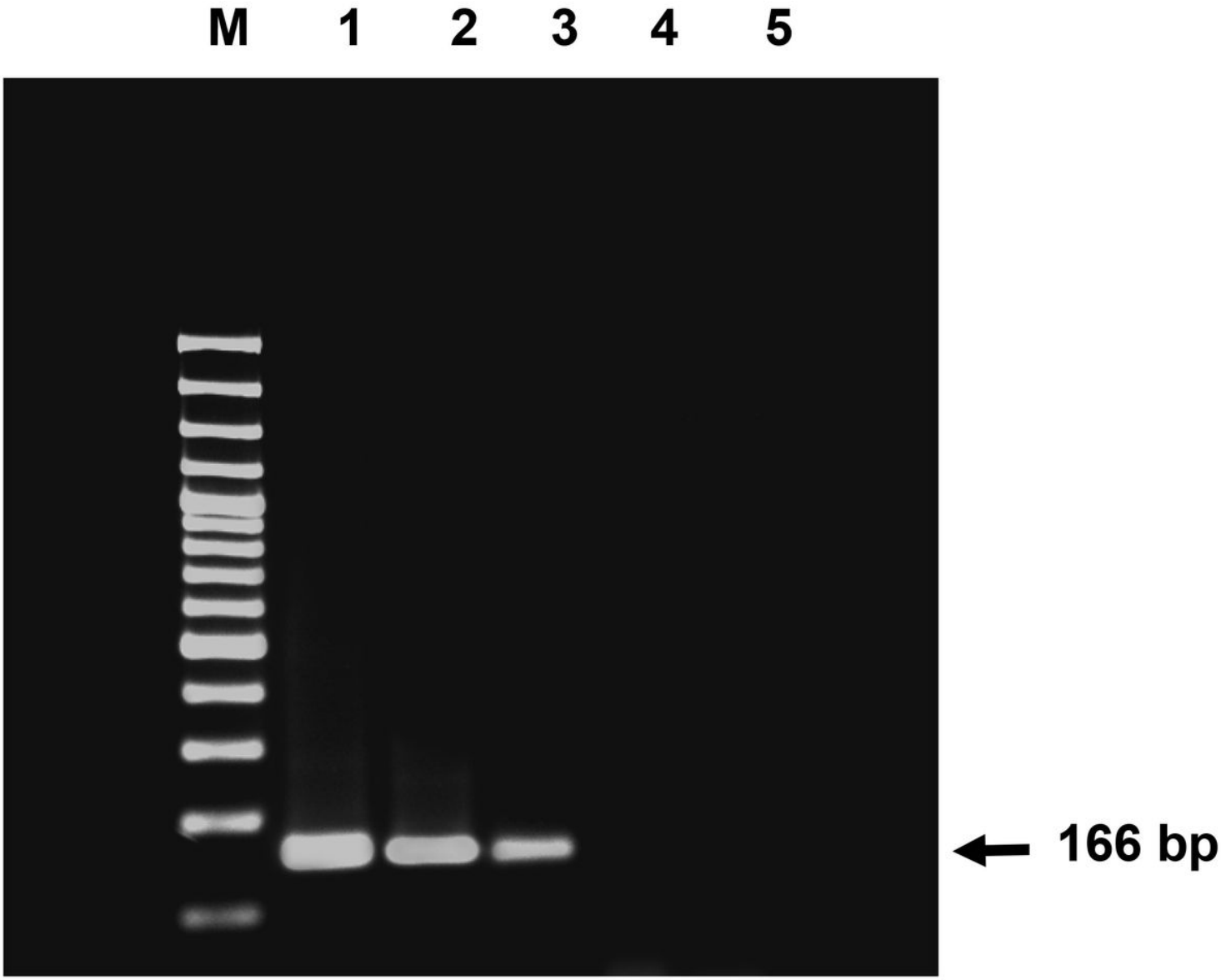

Figure 3

The detection limit of nested PCR assay for E. coli DNA using ten-fold serial dilutions of E. coli genomic DNA. Lane M, 100-bp ladder DNA marker; lane 1, 100 pg of DNA; lane 2, 10 pg of DNA; lane 3, 1 pg of DNA; lane 4, 0.1 pg of DNA, and lane 5, a reaction without DNA as a negative control.

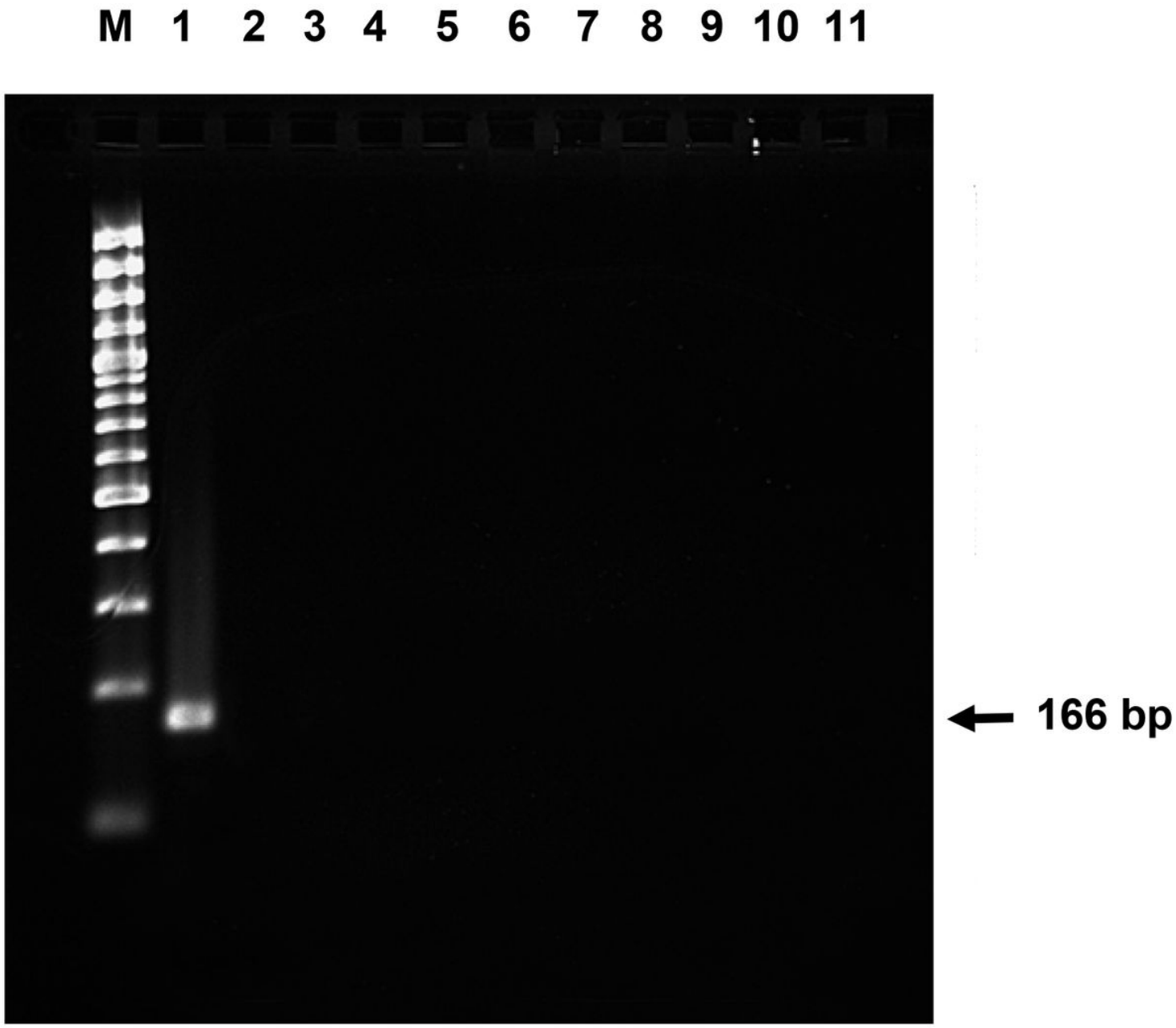

Figure 4

The detection limit of nested PCR assay for E. coli DNA using ten-fold serial dilutions of E. coli genomic DNA. Lane M, 100-bp ladder DNA marker; lane 1, 100 pg of DNA; lane 2, 10 pg of DNA; lane 3, 1 pg of DNA; lane 4, 0.1 pg of DNA, and lane 5, a reaction without DNA as a negative control.

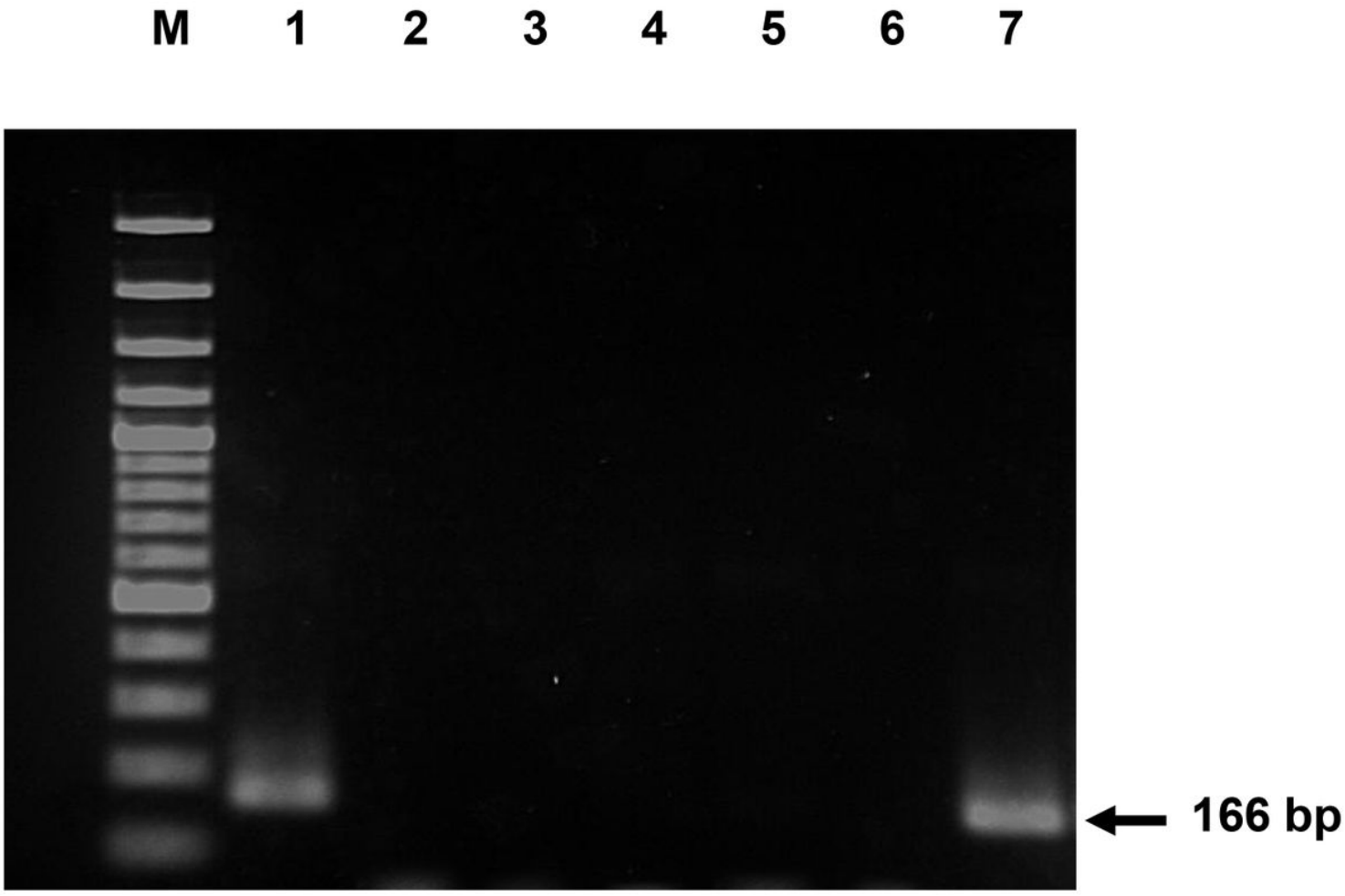

Figure 5

Detection of E. coli in stool samples using nested PCR assay. Lane M, 100-bp ladder DNA marker; lane 1, E. coli DNA; lane 2, negative control; lanes 3 to 6, negative samples for E. coli; lane 7, E. coli positive sample.

Supplementary Files

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