Construction of multiplex RT-PCR to determine the expression of ZO-1, Claudin-1, and Occludin genes in pig's intestine

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ARTICLE INFO

Research Paper

Received: August 31, 2024
Revised: November 18, 2024
Accepted: November 20, 2024

Keywords

Claudin-1
mRT-PCR
Occludin
pig
ZO-1

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ABSTRACT

Tight junction (TJ) proteins play a critical function in forming a strong intestinal barrier that protects against ingested pathogens and harmful agents. This study aimed to utilize multiplex RT-PCR (mRT-PCR) to assess the expression of ZO-1, Claudin-1, and Occludin genes at mRNA level in the intestines of pigs using specific primer pairs yielding amplicons of 167 bp, 500 bp, and 235 bp, respectively. The mRT-PCR protocol was optimized for annealing temperature and primer concentrations, including primer specificity, and determining the limit of detection. Subsequently, the optimized mRT-PCR was applied to detect these genes in 48 pig intestinal samples, including duodenum, jejunum and ileum. The mRT-PCR demonstrated specificity for these genes with the annealing temperature at 58°C. The primer pair ratio for ZO-1, Claudin-1, and Occludin was 0.4 µM:0.4 µM:0.4 µM (2:2:2). The detection rate for ZO-1, Claudin-1, and Occludin genes were 83.33% (40/48), 29.17% (14/48) and 4.17% (2/48) respectively. Intriguingly, one sample tested positive for all three mRNA, while negative results were observed in 12.5% of the samples. In conclusion, in the present study, the mRT-PCR was successfully established to detect ZO-1, Claudin-1, and Occludin expression in pig intestinal tissues.

Cited as: Nguyen, N. T. Q., Le, T. T. Q., Dang, N. V., Nguyen, M. M. T., Dong, T. T., Nguyen, T. Q., & Dinh, P. X. (2024). Construction of multiplex RT-PCR to determine the expression of ZO-1, Claudin-1, and Occludin genes in pig's intestine. *The Journal of Agriculture and Development* 23(Special issue 2), 110 -118.

1. Introduction

The intestinal mucosa is crucial for protecting the intestinal epithelium by preventing toxic substances and pathogens from entering the body. It consists of three layers: the mucosal epithelium, connective tissue, and muscle. The mucosa regulates nutrient intake, absorbs molecules, and maintains mucus thickness while eliminating toxins. However, when the intestinal barrier is compromised, it can lead to bacterial translocation, increased disease susceptibility, and impaired nutrient absorption, which negatively impacts pigs' health and the pig's industry (Wijtten et al., 2011).

The ZO-1 gene encoded the ZO-1 protein, a peripheral membrane protein with a mass of 210 to 225 kDa that is a scaffolding protein that links transmembrane proteins such as claudin and occludin to the cytoskeleton, playing a crucial role in strengthening junctions and maintaining intestinal barrier integrity (González-Mariscal et al., 2000). ZO-1 was vital for the structural integrity of the intestinal epithelium. Changes in ZO-1 activity can weaken tight junctions, leading to increased intestinal permeability, known as "leakage" which can result in conditions like enteritis (Sheth et al., 2000). The Occludin gene encodes the occludin protein, which is essential for tight junctions in epithelial and endothelial cells, with a molecular weight of 60 - 65 kDa (Otani & Furuse, 2020). Occludin consists of two extracellular and two intracellular (transmembrane) domains that regulate tight junction stability (González-Mariscal et al., 2000). Occludin directly interacts with the zonula occludin proteins ZO-1, ZO-2, and ZO-3, which are vital for the stability of the junction complex. The first half of occludin binds to ZO-1, helping maintain epithelial cell polarity necessary for optimal intestinal function and nutrient transport. Occludin indirectly interacts with the actin cytoskeleton and junctional adhesion molecule (JAM) via the ZO protein (Sheth et al., 2000). Claudin-1 gene was discovered in 1998 by Mikio Furuse and Shoichiro Tsukita in Japan through a cDNA library screening aimed at identifying unknown tight junction (TJ) components (Furuse et al., 1998). Claudin-1 is a transmembrane protein weighing 20 - 27 kDa, featuring four transmembrane domains and creating an "electrostatic filter" that regulates the movement of ions and enhances intercellular adhesion, crucial for the barrier function of skin and epithelial tissues (Anderson & Van Itallie, 2009). Although claudins share structural similarities with occludin, they lack sequence homology (Shin et al., 2006).

In this study, an mRT-PCR was constructed to detect the expression at mRNA level of these genes in intestinal samples. This process involves two reactions: reverse transcription of the target mRNA molecules into cDNAs, followed by PCR to amplify the cDNAs. In this study, an mRT-PCR was constructed to detect the expression of ZO-1, Claudin-1, and Occludin gene at mRNA level from pig intestine samples to support for the further diagnostic conditions.

2. Materials and Methods

2.1. Primers for multiplex RT-PCR

Primer pair detecting ZO-1, Occludin and Claudin-1 (167 bp, 235 bp, and 500 bp, respectively) were obtained from published documents with minor modification and shown in Table 1. The primers were checked by Primer3Plus and NCBI Blast Primer tools and synthesized by IDT (Integrated DNA Technologies).

Table 1. Primers were used in this study

Gene	Sequence 5' - 3'	Length	Amplicons	References
ZO-1	F: GGATGGTCACACCGTG	16	167 bp	(Liu et al., 2017)
	R: GGAGGATGCTGTTGTCTC	18		
Claudin-1	F: AAGATTTACTCCTACGCTGGT	21	500 bp	(Zhang et al., 2018)
	R: CTTGGTGTTGGGTAAGATG	19		
Occludin	F: ACGAGCTGGAGGAAGACTGGATC	17	235 bp	(Luo et al., 2017)
	R: TGAGCCGTACATAGATCCAGAAGC	23		

2.2. Field samples and control samples

Forty-eight pig's intestinal samples, comprising 16 duodenum, 16 jejunum colon, and 16 ileum samples, were obtained from pig farms in Dong Nai and Binh Duong Provinces. The KT-Biotech Accutive pDNA/RNA Prep Kit was used to extract mRNA according to manufacturer's recommendation, then mRNA was kept at -20°C for further use.

Positive control was selected by choosing the field samples positive for all three mRNAs of ZO-1, Claudin-1 and Occludin by mRT-PCR and confirmed by sequencing method. Our positive control showed sequence identities up to 99.40%, 95.61%, and 100%, respectively when compared to Accession number AJ318101.1, NM_001161635.1, and NM_001163647.2.

2.3. Two-step mRT-PCR

cDNA was synthesized by RevertAid First Strand cDNA Synthesis Kit (#K1622, Thermo Scientific) and kept at -20°C for later uses.

Each mRT-PCR reaction contained 12.5 µL of DreamTaq Green PCR Master Mix (Cat# K1081, Thermo Scientific), primer mix with an initial concentration of 20 µM, cDNA of 3 µL and DEPC (Cat# K1081, Thermo Scientific) - treated water added to a total volume of 25 µL. The thermal cycling was pre-denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 95°C for 30 sec, annealing temperature 95°C for 30 sec, extension at 72°C for 40 sec and final extension at 72°C for 7 min. Next, amplification products were analyzed by electrophoresis on a 1.2% (w/v) agarose gel in 0.5X Tris-Acetate-EDTA (TAE) buffer (Cat#B49, ThermoFisher) containing Midori Green Advance DNA dye (Cat#MG04, Nippon Genetics). 1Kb plus DNA labeling (Cat#10787018, Invitrogen) was added to each DNA gel to indicate PCR product size and analyzed by UV transillumination.

2.4. Optimization of the primer concentration for the mRT-PCR assay

In order to optimize primer concentration of mRT-PCR, different primer concentrations for the three genes were tested, including ZO-1:Claudin-1:Occludin at 1:1:1 (0.2 µM:0.2 µM:0.2 µM); 1:2:1 (0.2 µM:0.4 µM:0.2 µM); 1:2:2 (0.2 µM:0.4 µM:0.4 µM); 2:2:2 (0.4 µM:0.4 µM:0.4 µM), 2:3:2 (0.4 µM:0.6 µM:0.4 µM), 2:4:2 (0.4 µM:0.8 µM:0.4 µM). Amplification results analyzed on agarose gel figured out the optimal concentration.

2.5. Evaluation of the specificity of the mRT-PCR assay

The specificity of primers used in the study were first checked through reliable information sites such as NCBI-BLAST and Insilico tool. Furthermore, the specificity of the primers was experimented with DNA extracted from unrelated microorganism such as *Salmonella, E. coli, Clostridium perfringens, Staphylococcus* spp., and ASF virus.

2.6. Determination of the detection limit of the mRT- PCR assay

Pure PCR products of each gene were recovered from low-melting agarose gel using the TopPURE ® Tissue Viral Extraction kit and the DNA concentration of each gene was adjusted to 1 ng/µL by diluting with TE buffer (1X). Subsequently, of the mixed samples was diluted in a 10-fold series from 1 ng/µL to 10^{-9} ng/µL. The lowest concentration produced visual band on an agarose gel would be considered as the limit of detection (LOD) of the mRT-PCR assay.

2.7. Application of the established mRT-PCR assay on field samples

The optimized mRT-PCR was applied on 48 intestine samples. The detection rate for each gene expression was determined to calculate the applicability of the assay.

3. Results

3.1. Optimal conditions of two-step mRT-PCR

To make sure the primer pairs function well, single RT-PCR was conducted at temperatures ranging from 56°C to 62°C for the ZO-1, Claudin-1, and Occludin genes, which have target sizes of 167 bp, 500 bp, and 235 bp, respectively. Electrophoresis demonstrated that the primers successfully amplified the expected mRNA at all tested temperatures: 56°C, 58°C, 60°C, and 62°C (Figure 1A-C). Among these, 58°C yielded the best amplification for all three genes and thus this temperature was used in mRT-PCR (Figure 1D). Later on, 58°C was selected as the optimized annealing temperature for the mRT-PCR assay.

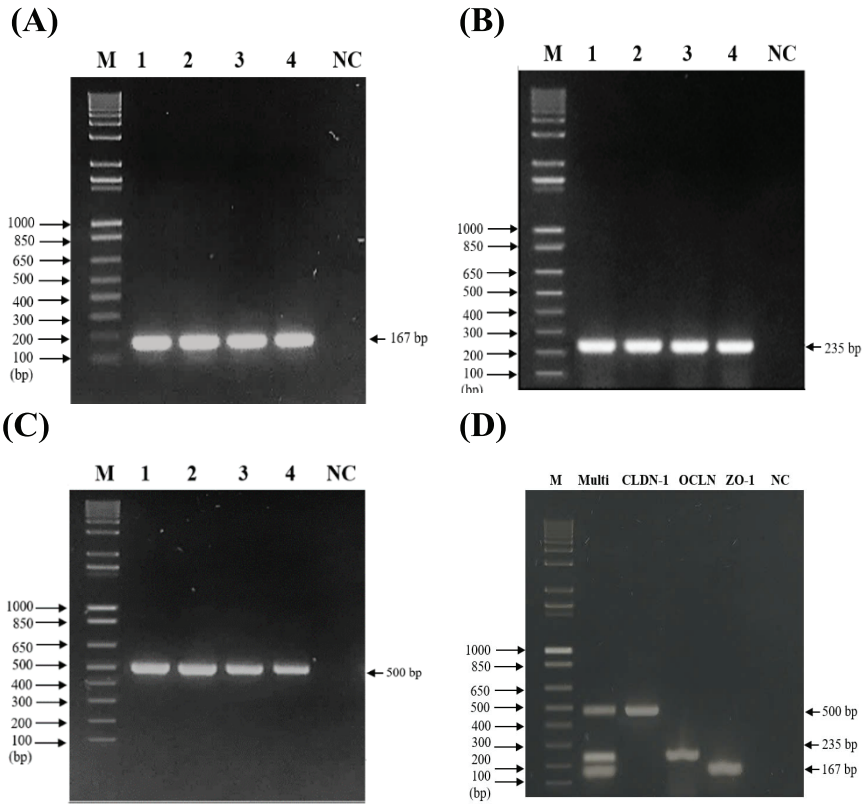

Figure 1. Results of investigation of optimal annealing temperature. A. The sRT-PCR detected ZO-1 gene (167 bp); B. The sRT-PCR detected Occludin (235 bp); C. The sRT-PCR detected Claudin-1 (500 bp); D. The mRT-PCR detection ZO-1, Claudin-1, and Occludin genes at 58°C. Lane M: DNA ladder 1 Kb plus; Lane 1: 56°C, Lane 2: 58°C, Lane 3: 60°C, and Lane 4: 62°C. Lane NC: negative control with nuclease-free water.

Next, the primer concentration of mRT-PCR was optimized. Figure 2 showed that the amplification effectiveness varies significantly when primers concentrations changed. The concentration ratio of 2:2:2 (0.4 µM:0.4 µM: 0.4 µM) in lane 4 produced equal and bright bands and was chosen as the concentration for subsequent reactions.

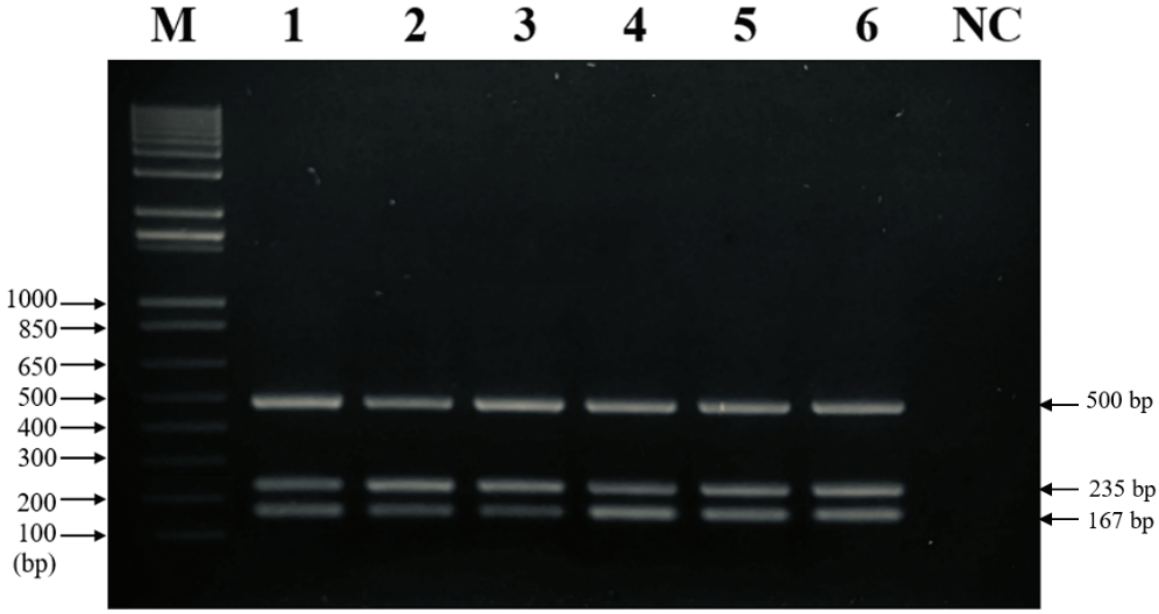

Figure 2. Electrophoresis results of optimizing of the primer concentration ratio of Claudin-1 (500 bp), Occludin (235 bp) and ZO-1 (167 bp). Lane M: 1 Kb Plus DNA marker, Lane 1: 1:1:1 (0.2 µM:0.2 µM:0.2 µM), Lane 2: 1:2:1 (0.2 µM:0.4 µM:0.2 µM), Lane 3: 1:2:2 (0.2 µM:0.4 µM:0.4 µM), Lane 4: 2:2:2 (0.4 µM:0.4 µM:0.4 µM), Lane 5: 2:3:2 (0.4 µM:0.6 µM:0.4 µM), Lane 6: 2:4:2 (0.4 µM:0.8 µM:0.4 µM); Lane NC: negative control with nuclease-free water.

3.2. Specificity and limit of detection of two-step mRT-PCR

To confirm the ability of accurate amplification of the primers in mRT-PCR, different DNA/RNA from unrelated bacteria and virus that are commonly found in environment and potentially contaminate the samples were used, including *Salmonella*, *E. coli*, *Clostridium*, *Staphylococcus* spp., African Swine Fever (ASF) virus. DNA from ducks and chickens were also added to the check list. Figure 3 confirmed that the positive control mRT-PCR reaction produced the expected bands at the correct sizes, while there was no amplification occurring for the unrelated DNA/RNA template (Figure 3). This result demonstrated that the mRT-PCR specifically detected ZO-1, Claudin-1, and Occludin mRNA and the primer pairs did not bind nonspecifically onto some common microorganism investigated.

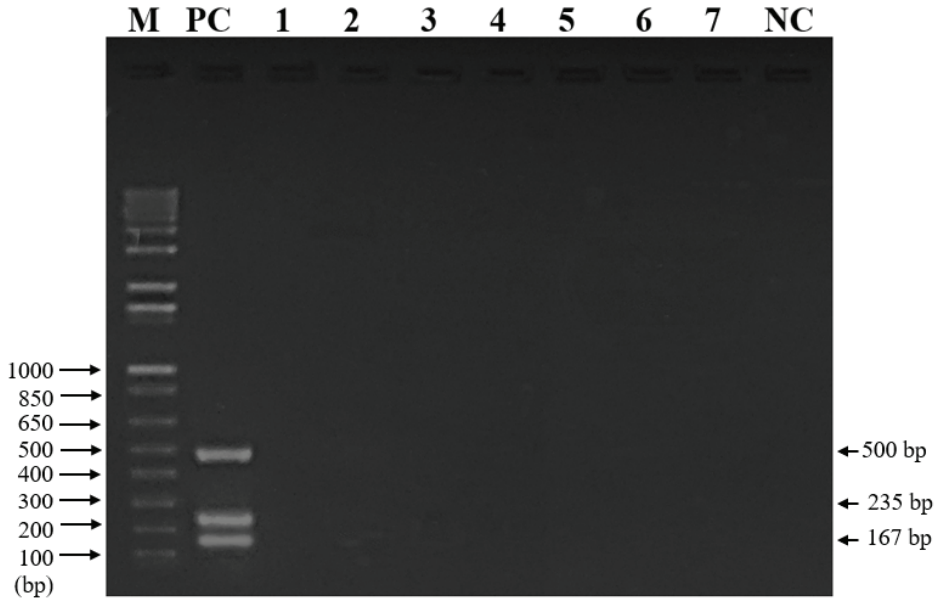

Figure 3. Electrophoresis results of testing the specificity of the mRT-PCR. Lane M: Ladder 1 Kb plus DNA, Lane PC: Positive control, Lane 1: *Salmonella*; Lane 2: *E. coli*, Lane 3: *Clostridium*; Lane 4: *Staphylococcus spp*, Lane 5: Chicken intestines; Lane 6: duck intestines, Lane 7: ASF; Lane NC: negative control with nuclease-free water.

To examine the detection limit of the mRT-PCR, the assay was investigated using template at various concentration, up to 10^{-9} ng/ µL. Figure 4 illustrated that the mRT-PCR could detect all the three target mRNA simultaneously in lane 6, corresponding to the concentration of 10^{-4} ng/ µL. The experiment was reproducible in triplicate which indicating that the detection limit of the mRT-PCR was 10^{-4} ng/ µL per reaction for each target mRNA.

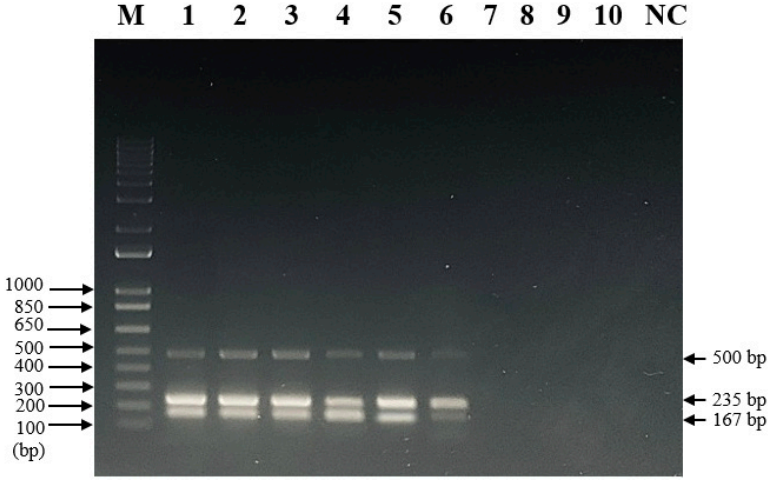

Figure 4. Determining the detection limit of mRT- PCR. Lane M: Ladder 1 Kb plus DNA, Lane 1: 1 ng/µL, Lane 2: 10^{-1} ng/µL, Lane 3: 10^{-2} ng/µL, Lane 4: 10^{-3} ng/µL, Lane 5: 10^{-4} ng/µL, Lane 6: 10^{-5} ng/µL, Lane 7: 10^{-6} ng/µL, Lane 8: 10^{-7} ng/µL, Lane 9: 10^{-8} ng/µL, Lane 10: 10^{-9} ng/µL, Lane NC: negative control with nuclease-free water.

3.3. Application of the optimized mRT-PCR assay to pigs intestinal samples

The optimized mRT-PCR was used to investigate 48 pig intestine samples (16 duodenum, 16 colons, and 16 ileum samples) collected pig farms in Dong Nai and Binh Duong Provinces to determine the expression of ZO-1, Claudin-1, and Occludin genes. Results showed that the positive rate of ZO-1 mRNA in the duodenum was 81.25% (13/16), jejunum was 75% (12/16) and ileum was 93.75% (15/16). For Occludin, its mRNA was detected in 12.5% (2/16) of ileum but not detected in duodenum and jejunum (0/16). For Claudin-1, its mRNA was found in 25% (4/16) of the duodenum, in 43.75% (7/16) of jejunum and 18.75% (3/16) of ileum. The positive rates of each mRNA in the total number of samples were 83.33% (40/48), 4.17% (2/48) and 29.17% (14/48), respectively for ZO-1, Occludin and Claudin-1. Table 2 shows that the positive rate of the ZO-1 gene was higher than that of Claudin-1 and Occludin genes in all three locations: duodenum, jejunum, and ileum. Furthermore, there was one sample positive for all three genes, accounting for 2.08% and the negative rate for all three genes was 12.5% (6/48). Therefore, the mRT-PCR reaction could be used to determine the presence of these genes in field samples (Figure 5).

Table 2. Detection rate of the three gene expression

Sample type	Number of samples	ZO-1 mRNA n (%)	Claudin-1 mRNA n (%)	Occludin mRNA n (%)
Duodenum	16	13 (81.25)	4 (25)	0 (0.00)
Jejunum	16	12 (75)	7 (43.75)	0 (0.00)
Ileum	16	15 (93.75)	3 (18.75)	2 (12.5)
Total	48	40 (83.33)	14 (29.17)	2 (4.17)

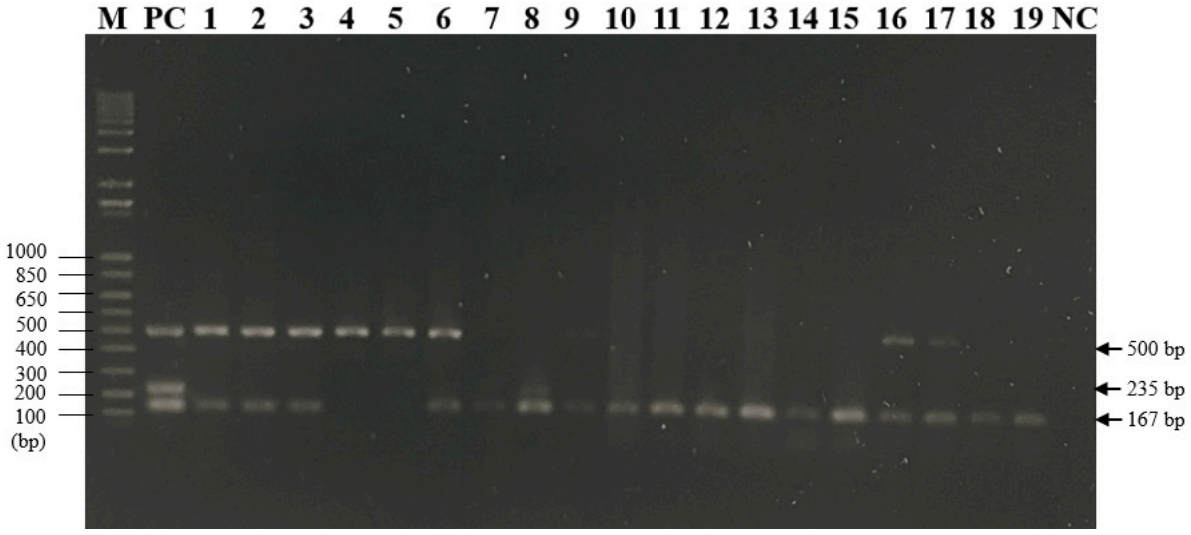

Figure 5. Detection of ZO-1, Claudin-1, and Occludin mRNA in intestinal samples. Lane 1-16: T1.1TT - T2.2TT; Lane 17 - 19: T2.2KT - T2.3TT; M: Ladder 1kb plus DNA, PC: Positive control, Lane NC: negative control with nuclease-free water.

3.4. Sequencing analysis

To evaluate the accuracy of the primer's pairs, the amplified product was sequenced. The sequencing results were analyzed on the BioEdit 7.2 and NCBI blast.

The amplicon of ZO-1 gene (sample T2.4HT) was 167 nucleotides and shared 99.40% similarity to the reference sequence AJ318101.1. Similarly, 500 nucleotides of Claudin-1 shared 95.61% identity with the reference sequence NM_001161635.1. Lastly, PCR product of Occludin gene showed 100% similarity at nucleotide level to the reference sequence NM_001163647.2.

4. Discussion

Tight Junction (TJ) family members like ZO-1, Claudin-1, and Occludin are vital in maintaining the intestinal barrier's integrity and protecting the body against pathogen invasion. Determining the expression of these gene can help evaluate the strength of the intestinal mucosa in different nutritional conditions or stress situation in animals.

In this study, we effectively established a Multiplex RT-PCR technique for simultaneously detecting the expression of ZO-1, Claudin-1, and Occludin genes in pig's intestinal tissue. The ZO-1 gene had the highest detection rate (83.33%), followed by the Claudin-1 and Occludin genes at 29.17% and 4.17%, respectively. In contrast, Dong et al. (2019) demonstrated that normally Occludin was expressed more than ZO-1, but when the pigs were fed with ZnO in the diet, the expression of ZO-1 was upregulated compared to Occludin. It indicated that the detectivity of these genes is dependent on the host's physiological conditions and the nutritional status. Unfortunately, in our study, due to lacking of the information about the nutritional condition, it was unable to figure out why ZO-1 was the most expressed in the samples.

In addition, the expression of these genes also depends on the location in the intestine. For example, the highest detection rate of the ZO-1 mRNA was in ileum (93.75%), while Occludin mRNA was barely detected in the duodenum and ileum. Other research revealed that Occludin level in pig intestinal cells was particularly susceptible to downregulation during stress conditions (Wu et al., 2020). These findings further highlight the expression of these three genes depends on various factors such as nutrition, stress, environmental conditions as well as physiological status of the animals.

5. Conclusions

The multiplex RT-PCR reaction was successfully optimized to determine the expression of ZO-1, Claudin-1 and Occludin at mRNA level in pig intestine. Optimal primer concentration ratio was 2:2:2 (0.4 µM:0.4 µM:0.4 µM). mRT-PCR functions well at annealing temperature of 58°C, and its detection limit was 10^{-4} ng/µL. The sequencing results of PCR amplification products for the ZO-1, Claudin-1 and Occludin genes confirmed the accuracy and reliability of the mRT-PCR for application in the field samples.

Conflict of interest

The authors have no conflict of interest related to this publication.

Acknowledgements

The author would like to express their deep gratitude to Nong Lam University, Ho Chi Minh City, Vietnam for funding this study.

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