Comparison of SYBR Green real time PCR assay and conventional PCR for identity of some commercial live poultry veterinary vaccines

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A B S T R A C T

Escherichia coli (E. coli) continue to be one of the major causes of food poisoning in the world. Different methods have been developed in order to reduce the time for the evaluation of the E. coli vaccines. *Infectious bronchitis (IB)* is a highly contagious viral disease of poultry causes economic losses. Control of IB virus has been attempted using live attenuated and inactivated vaccines. Due to the continuous emergence of E. coli and infections bronchitis, it was important to find a rapid accurate method of evaluation of the used live attenuated E. coli and IB vaccines. In this study, three assays, namely a conventional identification method including; Specific Pathogen Free eggs (SPF) eggs inoculation for IB vaccine and culture method for E. coli vaccine, conventional polymerase chain reaction (PCR) assay and SYBR Green I Real-Time PCR method were developed and evaluated on 10 fold serial dilutions of each vaccine. A comparative analysis of these three assays was then performed, and the results indicated that the SYBR Green I Real-Time PCR had the highest sensitivity and specificity.

Keywords: E. coli vaccine, IB vaccine, conventional PCR, Real-Time PCR

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1. INTRODUCTION

E. coli is a major cause of food poisoning in the world. Different techniques have been developed in order to reduce the time for the evaluation of the E. coli vaccines, since the International Organization for Standardization (ISO) standard cultural method requires up of five days. Many of the PCR assays employ either visual scoring of ethidium bromide-stained agarose gels or post-PCR hybridization-capture methods that are labour intensive, time consuming and difficult to automate. To date, the use of SYBR Green I for the identification of PCR product allows an early and simple approach to the Real-Time PCR and require less knowledge than classic Real-time PCR using fluorogenic oligoprobes (Hoorfar et al., 2000).

Infectious bronchitis (IB) is a highly contagious viral disease of poultry caused by infectious bronchitis virus (IB), a member of the genus Gammacoronavirus, family Coronaviridae, order Nidovirales.

This disease is characterized by respiratory signs and nephritis, leading to the yield of both meat-type and egg-laying birds being affected (ICTV virus taxonomy 2009). Thus, IB causes considerable economic losses within the poultry industry, and therefore, IB virus is considered as a major pathogen in poultry production (OIE 2018.). The IB virus genome consists of a single-stranded positive-sense RNA molecule of approximately 27.6 kb that encodes several nonstructural proteins involved in RNA transcription and replication and four structural proteins: small membrane (E), membrane (M), nucleoprotein (N) and spike (S), which is formed by a globular S1 subunit that is anchored in the membrane by the S2 subunit (Cavanagh 2007).

Since IB virus was first described by Schalk and Hawn in the 1930s (Schalk and Hawn 1931), many serotypes have been identified worldwide. Control of IB virus has been attempted using live attenuated and inactivated vaccines. Due to the continuous emergence of new variants of the IB virus, the identification of the type of IB virus causing an outbreak in commercial poultry is important in the selection of the appropriate vaccine(s) capable of inducing a protective immune response (Ana et al., 2013). From this point it was important to find a rapid accurate method of evaluation of the used live attenuated IB vaccines.

The fluorescent-probe-based assays require availability of primers and probes that must be selected according to very rigid conditions, which cannot always be easily applied. Use of the double stranded DNA (dsDNA) binding dye SYBR Green I for identification of PCR products has overcome this limitation by allowing real-time PCR to be applied without the need for probes linked to fluorescent molecules Protocols that are already in use for classic PCR can thus be used with only slight modifications. In the absence of probes, specificity of the reaction is determined by melting temperature (Tm) of the amplicon obtained, defined as the temperature at which 50% of the DNA amplicon is in a double stranded configuration (Hyang-Mi et al., 2005). Recently, more researchers rely upon real-time PCR studies using simple and less expensive SYBR Green dye. A number of SYBR Green real-time PCR assays for identification of microbial pathogens such as Escherichia coli O157:H7 have been reported. (Jothikumar and Griffiths, 2002)

The objective of the present study was to develop and evaluate a SYBR Green I Real-Time PCR method for the specific identification and identification of live attenuated vaccines that would be suitable for routine analysis of these vaccines samples.

To confirm the effectiveness of the proposed method experiments were conducted with two different live vaccines. The current study was undertaken to develop a simple, inexpensive, sensitive and specific SYBR Green I quantitative real-time PCR method for separate identification of E. coli and IB in live attenuated vaccines.

In this study, three assays, namely a conventional identification method (cultural method for E. coli and SPF inoculation for

IB virus), conventional PCR assay and SYBR Green I Real-Time PCR method were compared for their efficiency for rapid accurate identification of E. coli and IB virus. A comparative analysis of these three assays was then performed, and the results indicated that the SYBR Green I Real-Time PCR had the highest sensitivity and specificity, with an advantage of fast turnaround over the other two assays, which is a valuable feature for identification and identification of E. coli and IB live vaccines.

2. MATERIAL AND METHODS

2.1. Conventional culture method for identification of E. coli on plate media:

Fourteen, serial 10-fold dilutions of E.coli commercial live attenuated vaccine were prepared in Tryptic soy broth then plated onto MacConkey (Mc) agar then incubated overnight at 37°C .To rule out false positives, one uninoculated aliquot was used as a negative control in every experiment the method was applied following the standard procedures of the World Organization for Animal Health (OIE 2018) to determine the lower identification limit of the Conventional culture method.

2.2. SPF Egg inoculation for identification of IB virus in SPF eggs:

Different serial ten dilutions of the commercial live attenuated IB vaccine was prepared to be inoculated in 9-11day old specific pathogen-free embryonated chicken eggs (5 eggs for each dilution), following the standard procedures of the World Organization for Animal Health (OIE 2018) to show lesion of IB virus on egg embryo as it shows curling and dwarfing. The allantoic fluid was harvested and stored at-70oC until used for RNA extraction.

2.3. Conventional PCR for E. coli:

2.3.1. DNA extraction form E. coli:

DNA extraction from diluted E. coli vaccine samples was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. Briefly, 200 µl of the sample suspension was incubated with 10 µl of proteinase K and 200 µl of lysis buffer at 56°C for 10 min. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100 µl of elution buffer provided in the kit.

2.3.2. Oligonucleotide Primer:

Primers used were supplied from Metabion (Germany) are listed in table (1).

Table (1): E. coli Primers sequences, target gene, amplicon sizes:

Target gene	Primers sequences	Amplified segment (bp)	Reference
phoA	CGATTCTGGAAATGGCAAAAG CGTGATCAGCGGTGACTATGAC	720	Hu *et al*., 2011

2.3.3. PCR amplification for identification of E. coli:

Primers were utilized in a 25-µl reaction containing 24 µl of Emerald Amp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmol concentration, 4.5 µl of water, and 6 µl of DNA template. The reaction was performed in a Biometra thermal cycler. A primary denaturation step was done at 95 °C for 5 min, followed by 35 cycles of 94°C for 30 sec., 55°C for 40 sec. and 72°C for 45 sec. min. A final extension step was done at 72°C for 10 min.

2.4. Conventional PCR for IB virus:

2.4.1. RNA extraction for IB virus:

RNA extraction from samples was performed using the QIAamp viral RNA Mini kit (Qiagen, Germany, GmbH). Briefly, 140 µl of the sample suspension was incubated with 560 µl of AVL lysis buffer and 5.6 µl of carrier RNA at room temperature for 10 min. After incubation, 560 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 60 µl of elution buffer provided in the kit.

2.4.2. PCR amplification for detection of IB:

Primers (table 2) were utilized in a 25-µl reaction containing 12.5 µl of Quantitect probe Reverse transcriptase PCR buffer (QIAgen, Gmbh), 1 µl of each primer of 20 pmol concentration, 0.25 µl of Reverse transcriptase enzyme 4.25 µl of water, and 6µl of template. The reaction was performed in a Biometra thermal cycler. Reverse transcription was applied at 50°C for 30 min, a primary denaturation step was done at 95°C for 5 min, followed by 35 cycles of 94°C for 30 sec., 48°C for 40 sec. and 72°C for 45 sec. min. A final extension step was done at 72°C for 10 min.

Table (2): IB Primers sequences, target gene, amplicon sizes.

Target gene	Primers sequences	Amplified segment (bp)	Reference
S1	ACT ACT ACC AAA GTG CCT ACA TCT TGT GCA GTA CCA TTA ACA	570 bp	Abdel-Moneim et al., 2002

2.5. Analysis of the PCR Products:

The products of PCR were separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 15 µl of the products were loaded in each gel slot. Gelpilot 100 bp (Qiagen, Germany, GmbH) and a gene ruler 100 bp ladder (Fermentas, Germany) were used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software.

2.6. E. coli SYBR I green Real time PCR:

Primers (table 3) were utilized in a 25-µl reaction containing 12.5 µl of the 2x QuantiTect SYBR Green PCR Master Mix (Qiagen, Germany, GmbH), 0.5 µl of each primer of 20 pmol concentration, 8.5 µl of water, and 3 µl of RNA template. The

reaction was performed in a Stratagene MX3005P real time PCR machine.

2.7. IB SYBR green rt-PCR:

Primers (table 3) were utilized in a 25-µl reaction containing 12.5 µl of the 2x QuantiTect SYBR Green PCR Master Mix (Qiagen, Germany, GmbH), 0.25 µl of RevertAid Reverse Transcriptase (200 U/µL) (Thermo Fisher), 0.5 µl of each primer of 20 pmol concentration, 8.25 µl of water, and 3 µl of RNA template. The reaction was performed in a Stratagene MX3005P real time PCR machine.

Table (3): Primers sequences, target genes, amplicon sizes and cycling conditions for SYBR green RT-PCR.

Target		E. coli	IB
Primers sequences		CGATTCTGGAA ATGGCAAAAG	ACTACTACCAAA GTGCCT
		CGTGATCAGCG GTGACTATGAC	ACATCTTGTGCA GTACCATTAACA
Reverse transcription		-	50˚C 30 min.
Primary denaturation		94˚C 15 min.	94˚C 15 min.
Amplification (40 cycles)	Secondary denaturation	94˚C 15 min.	94˚C 15 min.
	Annealing (Optics on)	55˚C 30 sec.	48˚C 30 sec.
	Extension	72˚C 30 sec.	72˚C 30 sec.
Dissociation curve (1 cycle)	Secondary denaturation	94˚C 1 min.	94˚C 1 min.
	Annealing	55˚C 1 min.	48˚C 1 min.
	Final denaturation	94˚C 1 min.	94˚C 1 min.
Reference		Hu et al., 2011	Abdel-Moneim et al., 2002

3. RESULTS

The analytical sensitivity of these three assays was assessed through serial dilutions of each vaccine:

3.1. Determination of the least detectable concentration of live attenuated E. coli live vaccine in culture media:

Among the diluted vaccine samples eleven dilutions revealed positive E. coli colonies on Mac agar media.

3.2. Determination of the least detectable concentration of live attenuated IB live vaccine in SPF eggs:

Among the diluted vaccine samples seven dilutions revealed positive IB picture of embryos curling and dwarfing.

3.3. Conventional PCR:

For identification of E. coli and IB and discrimination of the lower detectable concentration than the conventional

identification method in live attenuated E.coli live vaccines. The 720 bp and 570bp amplicons specific to E. coli and IB, respectively, were detected by routine PCR with the specific primers (Table 1). The identification limit was showed in fig 1 and 2.

3.3.1. Identification of E. coli:

The results of PCR for the amplification of the different dilutions of E. Coli live attenuated vaccine were positive at an amplification of 720 bp specific for the Genus E. Coli for the dilutions from the 12th to 27th but the 28th ,29th and 30th were negative.

3.3.2. Identification of IB:

PCR targeted a sequence corresponding to the IB virus. The obtained PCR products of 570 bp in length were separated on a 1.5 % agarose gel stained with ethidium bromide (Fig. 2). The results of PCR for the amplification of the different dilutions of IB live attenuated vaccine were detectable at an amplification of 570 bp specific for the dilutions from the 8th to 30th but the dilutions from 31th to 35th were negative.

3.4. Specificity of SYBR green I real-time RT-PCR:

For identification of E. coli and IB and discrimination of the lower detectable concentration than the conventional PCR assay in live attenuated E. coli and IB vaccines.

3.4.1. Identification of E. coli:

The amplification curve showed that 28^{th} and 29^{th} dilutions were considered as positive and dilution 30^{th} was considered as negative (fig 3A). Melting peaks analysis on the PCR products E. coli vaccine strains and tenfold serially diluted DNA did not indicate primer dimers or nonspecific products. Specific amplification of the E. coli target sequence was identified by the generation of a melt peak. The specificity of the SYBR green-I real-time RT-PCR was 100 % since detectable fluorescent signals was not observed with the negative control. Only the E. coli vaccine strain genetic material was detected (Fig. 3B).

3.4.2. Identification of IB:

The amplification curve showed that 31^{th} dilution was considered as positive but dilution 33^{th} and 35 was considered as negative (fig 4A).

Melting peaks analysis on the PCR products IB vaccine strains and tenfold serially diluted cDNA did not indicate primer dimers or nonspecific products. Specific amplification of the IB target sequence was identified by the generation of a melt peak. The specificity of the SYBR green-I real-time RT-PCR was 100 % since detectable fluorescent signals was not observed with the negative control. Only the IB vaccine strain genetic material was detected (Fig. 4B).

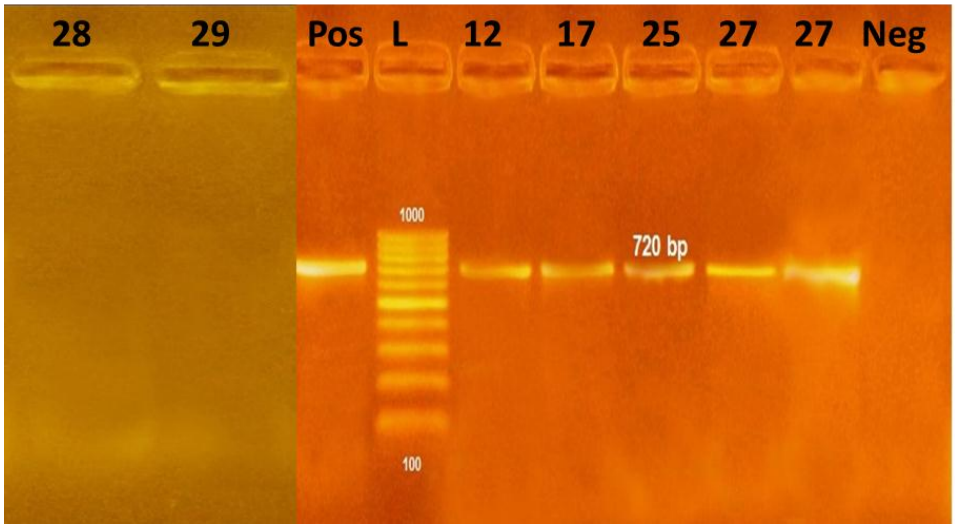

Figure 1. The sensitivity of conventional PCR assay for identification of E. coli.
Lane L. 100bp marker.
Lanes 12-27. positive amplification of 720bp fragment of E. Coli.
Lane pos. positive control E. Coli (ATCC 43 482).
Lane neg. negative control.
Lanes 28 and 29. Negative amplification of 720bp fragment of E. Coli of 28th and 29th dilutions.

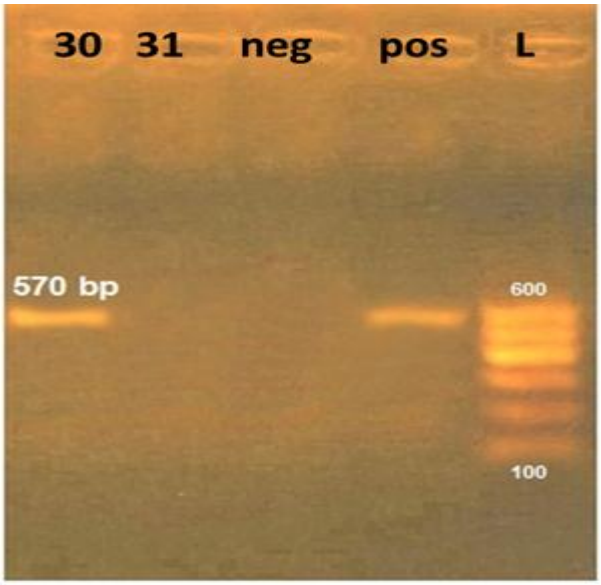

Figure 2. The sensitivity of conventional PCR assay for identification of IB virus.
Lane L. 100bp marker.
Lane pos. positive control IB (Access. No. AF395531).
Lane neg. negative control.
Lanes 31. Negative amplification of 570bp fragment of IB of 31th dilution.
Lanes 30. positive amplification of 570bp fragment of IB.

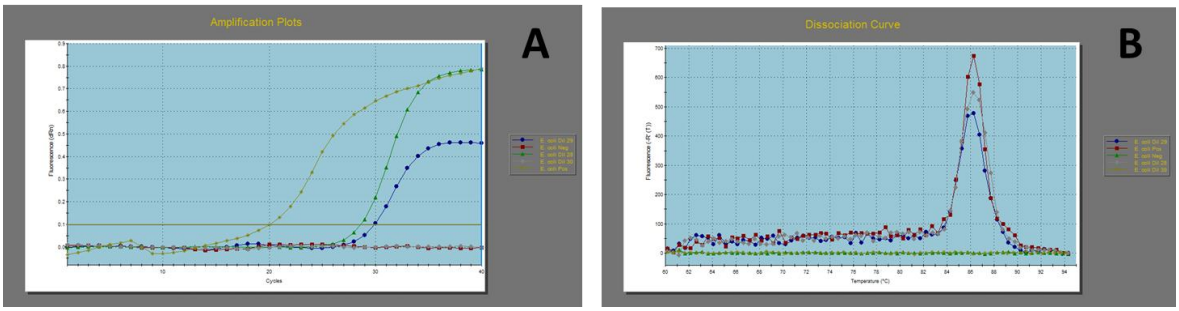

Fig. 3. (A) SYBR Green Real-Time PCR the specific fluorescent signals of E. Coli. (B) The dissociation curve analysis of SYBR Green real-time PCR product of E. Coli.

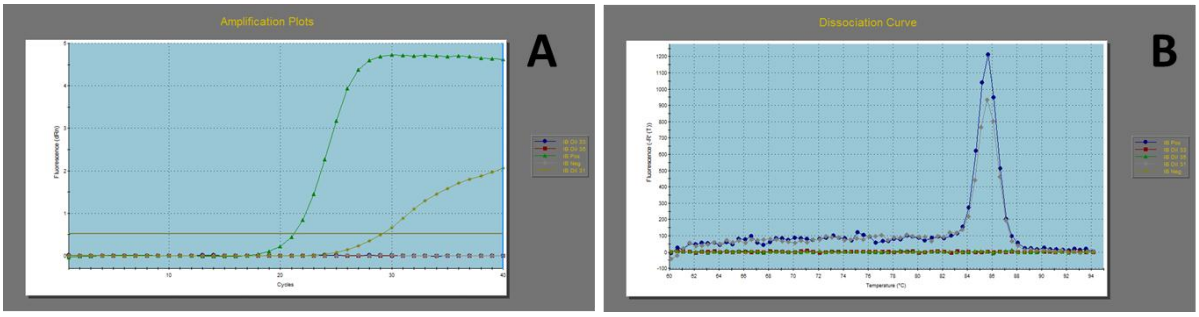

Fig. 4. (A) SYBR Green Real-Time PCR the specific fluorescent signals of IB. (B) The dissociation curve analysis of SYBR Green real-time PCR product of IB.

4. DISCUSSION

Virus isolation was a gold standard method for viral identification, but it is a time-consuming, labor-intensive work and has limitations. Up to the present time, there are several methods based on the conventional PCR and SYBR Green Real-Time PCR to detect and identify IB (Zhou et al., 2017). The use of PCR for diagnosis of viral diseases has increased to the point that the assay is now considered the gold standard instead of viral isolation. SYBR Green Real-Time PCR has catalyzed wider acceptance of PCR as a diagnostic tool because it is more rapid, sensitive, and reproducible, and the risk of carryover contamination is minimized compared to conventional PCR (Siham et al., 2016).

To be an alternative method, in this study, three assays for detecting the E.coli and IB in the live attenuated vaccines have been established and evaluated The SYBR Green Real-Time PCR is the most convenient method for the identification of E.coli and IB as it is highly sensitive(Zhou et al., 2017).

The results of using conventional identification of IB by SPF inoculation technique can detect IB until the 7^{th} dilution while the conventional culture method could detect E. coli until the 11^{th} dilution. The PCR assay could detect the virus RNA until the 30^{th} dilution and the bacterial DNA

until the 27th dilution but this method needs amplification product separations by gel electrophoresis. these are a time-consuming protocol. These results agree with (Ana et al., 2013) as he mentioned that Virus isolation (VI) is considered as the gold standard, but this method is time-consuming because several passages may be required to detect the virus. To overcome this drawback, different molecular assays have been developed.

On the other hand, SYBR Green I-based real-time PCR assays have proven to be one of the most effective tools in the rapid and sensitive identification of a variety of viral pathogens (Ana et al., 2013) as it was able to detect the virus until the 31th.

The SYBR Green I-based real-time PCR assay can detect very low numbers of bacterial cells or viral particles in vaccine samples even with high levels of background flora and common PCR inhibitors (Hyang-Mi et al., 2005).

The assay developed in the current study displayed an analytical sensitivity higher than the VI test or the conventional PCR assays. This last finding could be for a number of different reasons. First, SYBR Green I-based real-time based on the principle of identification of the fluorescent signal emitted by specific amplification products, has provided the assay with a higher sensitivity as compared with end point-conventional PCR. Second, viral isolation can be affected by the neutralizing action of the antibodies (Ana et al., 2013).

On the one hand, the SYBR Green I is less influenced by different genetic backgrounds. Thus, the use of SYBR Green I is considered as a broad-spectrum identification format. On the other hand, when using SYBR Green I as the format of identification, different amplicons can be distinguished by melting curve analysis. The melting curve for a specific PCR product is determined by the reduction in relative fluorescence when the double-strand of DNA is denatured by the temperature action. Therefore, the peak location on a melting curve (Tm) depends on the sequence, GC/AT ratio and the length of the resulting amplicon. The RT-PCR assay based on SYBR Green-I identification coupled with melting curves analysis for the identification of IB virus is proposed. The results indicate the usefulness of this tool for rapid identification of IB **(**Ana et al., 2013). It is also considered simpler and cheaper than other fluorescent dyes (Hairul et al., 2008).

To date, the approaches to detect E. coli and IB include conventional culture method; virus isolation and conventional PCR are time-consuming with relatively low specificity and sensitivity. Improved diagnostic assays with a broad identification spectrum for the identification of E. coli and IB are indeed needed.

It is concluded that SYBR Green I-based real-time RT-PCR assay provides a powerful tool for the identification of E. coli and IB. The proposed method seems to be effective, rapid and reproducible, showed that neither false-positive nor false-negative results were obtained. The specificity of the reaction was confirmed by the determination of the Tm, specific for the amplicon obtained, that allows to eliminate

the phase of electrophoresis, which is time-consuming and requires the use of ethidium bromide, a potent mutagenic agent, that is not suitable for routine use.

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