Using Multiplex-PCR In The Identity And Detection Of Extraneous Virus Contaminants Of Live Viral Vaccines Of Poultry

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ABSTRACT

In order to develop an *in vitro* method for the quality control of live Attenuated poultry viral vaccines for identity and the absence of extraneous agents Multiplex-PCR was applied for the identification of Infectious Bursal Disease virus vaccine (IBDVv) and Marek's (HVT) vaccine as well as detection of Chicken Anemia Virus (CAV) –with different titers– that experimentally contaminate each of the two vaccines .

Live attenuated IBDV vaccine -experimentally contaminated with CAV– was employed for the establishment of the method for the RNA derived viral vaccines .Both DNA and RNA extraction were carried out, then specific cDNA segment of the IBDVv was obtained after RT utilizing specific primer pair, followed by Multiplex-PCR using two primer pairs, the first for IBDVv and the second specific for CAV. On the other hand HVTV vaccine experimentally contaminated with CAV was employed for the establishment of the method for the DNA derived viral vaccines using two primer pairs, the first was specific for HVT virus and the second for CAV.

The Multiplex-PCRs for the two vaccines were highly specific and sensitive and could detect also the CAV (the experimental extraneous contaminant) at a detectable limit of 10^3 $TCID_{50}$/ 5µl of the sample. These results demonstrate the applicability of the method for the quality control of poultry vaccines for identity and for the absence of extraneous agents.

INTRODUCTION

Requirements for the quality control of poultry viral vaccines have increased considerably over recent years as a result of improved knowledge of potential risks associated with the use of these vaccines for the target birds themselves or for the consumers of these birds or their products. Vaccines are considered to be the most cost effective tool in the prevention of infectious diseases, particularly in poultry farming but they must be well identified and free of viral contaminants that could infect birds. Identity testing of a vaccine identifies the type of attenuated virus contained in such vaccine. Many traditional tests, FAT, SNT, and AGPT were indicated *(1)* under the title of general requirement for live viral vaccines. On the other hand purity testing has become an essential quality requirement of immunological veterinary medicinal products. The European pharmacopoeia *(2)* requires avian viral vaccines to be free of adventitious agents.

Tables 1 & 2 showed the conventional tests required for detection of extraneous agents in either seed lots and/or in batches of the final products **(2).** Under these texts seed material and final batches of poultry vaccines must be tested for a comprehensive list of potential contaminants (Table 2).

Table 1. The European pharmacopoeia regulatory frame work for extraneous agents testing of avian vaccines.

Methods of Analysis/Biological tests	Serial Number
"Nucleic acid amplification techniques"	2.6.21
"Avian Viral Vaccines: Tests for Extraneous Agents in Seed Lots"	2.6.24
"Avian Viral Vaccines: Tests for Extraneous Agents in Batches of Finished Products"	2.6.25
General Texts on Vaccines	
"Chicken Flocks Free from Specified Pathogens for the Production and Quality Control of Vaccines"	5.2.2
"Substances of Animal Origin for the Production of Veterinary Vaccines"	5.2.5
General Monograph	
"Vaccines for Veterinary Use"	0062

Table 2. Standard tests for extraneous agents in avian vaccines

Agent	Type of test
Avian adenovirus, group 1	SN, EIA, AGP
Avian encephalomyelitis virus	AGP, EIA
Avian infectious bronchitis virus	EIA, HI
Avian infectious laryngotracheitis virus	SN, EIA, IS
Avian leucosis viruses	SN, EIA
Avian nephritis virus	IS
Avian orthoreoviruses	IS, EIA
Avian reticuloendotheliosis virus	AGP, IS, EIA
Chicken anaemia virus	IS, EIA, SN
Egg drop syndrome virus	HI, EIA
Avian infectious bursal disease virus, Serotype 1 and 2	AGP, EIA, SN
Influenza A virus	AGP, EIA, HI
Mareks disease virus	AGP
Newcastle disease virus	HI, EIA
Turkey rhinotracheitis virus	EIA
Salmonella pullorum	AGG

Abbreviations: AGG = agglutination; AGP = agar gel precipitation; SN = serum neutralization; EIA = enzyme immunoassay (i.e. ELISA); HI = haemagglutination inhibition.

Other types of tests, nucleic acids amplification techniques "NAT" could be used for both identity testing and extraneous agent testing after validation for sensitivity and specificity *(2)* instead of the conventional tests that indicated in the identity testing and extraneous agent testing.

This study was planned to design Multiplex-PCR that could identify live attenuated poultry virus vaccine as well as detect specific extraneous viral contaminant as follow:

Identity testing of two poultry viral vaccines the first is an IBDVv (RNA virus) and the second HVT vaccine (DNA virus) separately.

CAV detection as an experimental extraneous virus contaminant in both vaccines at variable concentrations of that contaminant.

MATERIAL AND METHODS

Vaccines

Commercial IBDV and HVTV vaccines - that were previously evaluated in the Central Laboratory for Evaluation of Veterinary Biologics and pass the identity tests and proved to be free from extraneous viral contaminates- were used in this study for identity test. Each vial of each vaccine was diluted with sterile PBS. Commercial CAV vaccine was used to experimentally contaminate the IBDV and HVTV vaccines. The CAV vaccine as a source of viral contamination was serially diluted in each vaccine to produce 3 titers of 10^4, 10^3 and 10^2 per each PCR reaction of CAV.

Extraction of nucleic acid

DNA extraction

All the experimentally contaminated IBDV and HVTV vaccines were exposed to DNA extraction using Qiagen DNA extraction kit (Dneasy Blood & Tissue Kit) following the manufacturer's instructions.

RNA extraction

All the experimentally contaminated IBDV vaccines were subjected to RNA extraction using Qiagen RNA extraction kit (QiAamp Viral RNA Mini Kit) following the manufacturer's instructions.

RT

The extracted RNA of the IBDV vaccine were utilized in cDNA synthesis using specific primer pair *(3)*. cDNA synthesis kit of fermentaz company was used following the manufacturer's instructions (RevertAid First Strand cDNA Synthesis Kit) Table 3.

Table 3. The sequences of the selected primers

Virus	Genome	Code	5′-3′ Sequence	Fragment size
IBDV	RNA	IBDF	acaggcccagagtctaca	480 bp
		IBDR	Aycctgttgccactctttc	
HVTV	DNA	HVTF	cgcgtactgcgcctgacg	388 bp
		HVTR	caacttcgctcttagcg	
CAV	DNA	CAVF	ccgcacataccggtcggcagt	713 bp
		CAVR	ggggttcggcagcctcacactat	

PCRs (for identity test)

The processed cDNA of IBDV vaccine was utilized as a template in the amplification of 480 bp fragment specific for IBDV *(3)* with the use of Qiagen PCR kit (Taq PCR Master Mix Kit) following the manufacturer's instructions. The 50µl reaction volume consists of 25µl master mix 0.2 µM of each primer, in addition to the template DNA and nuclease free water. The cycling condition were ,initial Denaturation at 94 °C for 5 min., followed by 35 cycles of 94 °C for 1min. ,annealing at 55 °C for 1min. And extension at 72 °C for 1min. Followed by final extension at 72 °C for 7 min.

DNA of HVTV vaccine was utilized as a template to amplify 388 bp fragment specific for HVTV *(4)*. With the same reaction and cycling conditions (table 3).

Another 5 µl of the DNA of HVTV and 5µl of the experimentally contaminated IBDV were utilized in 2 separate PCRs using a pair of primers specific for CAV *(5)* to produce 713 bp fragment specific for CAV.(Table 3). The same reaction conditions and cycling conditions and primers concentrations were followed.

Multiplex-PCR

Five µl of DNA extraction from each dilution of the experimentally contaminated HVTV vaccines were utilized in this reaction using two primer pairs specific for HVTV and CAV vaccines. PCR kit of Qiagen was used according to the manufacturer's instructions. The cycling condition were, initial Denaturation at 94°C, followed by 35 cycles of 94 °C for 1min. ,annealing at 55°C for 1min. And extension at 72°C for 1min. Followed by final extension at 72°C for 7 min.

Five µl of DNA extracted from each dilution of the experimentally contaminated IBDV vaccine in addition to 5µl of the cDNA of IBDV were utilized in this reaction using two primer pairs specific for IBDV and CAV. Qiagen PCR kit was used following the manufacturer's instructions. With the same cycling condition as the previous method one.

RESULTS

Identity test

Both IBDV and HVTV were identified using PCR or/ and Multiplex-PCR utilizing the primer specific for each of them and the 480 bp and 388 bp fragment specific for IBDV and HVTV, were obtained (Fig.1) respectively.

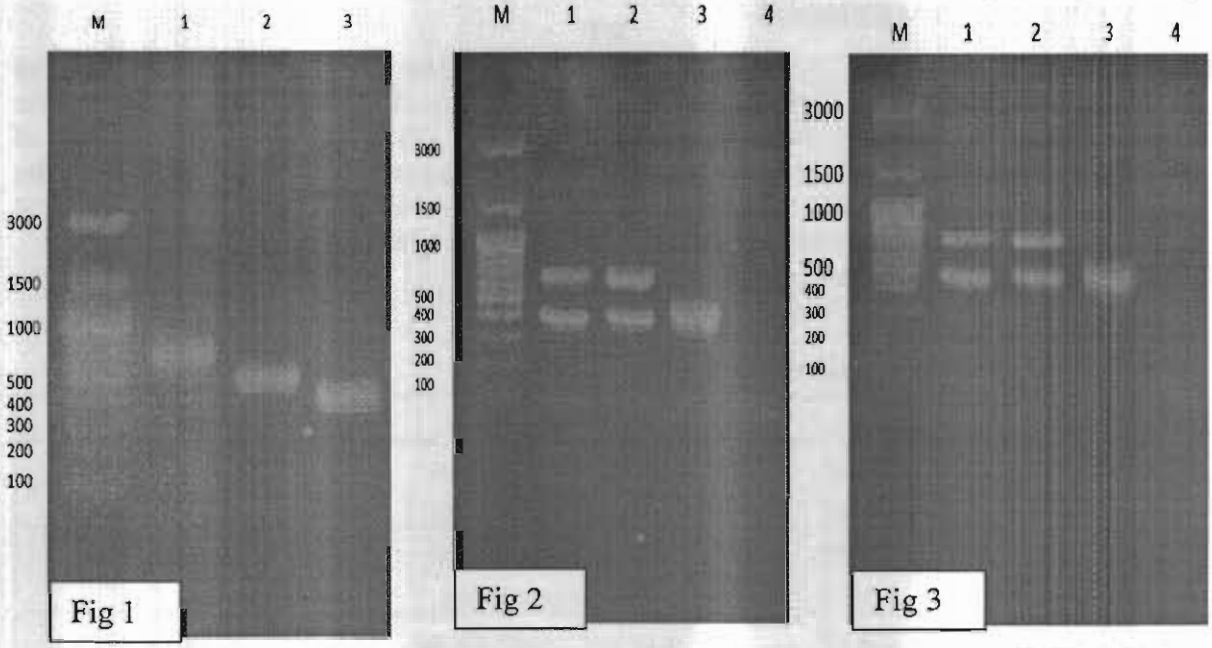

Fig. 1.Shows the results of PCR for identity of the CAV, IBDV & HVTV vaccines, Lane M :100 bp ladder; lanes 1-3:Shows fragments of 713,480 and 388 bp representing CAV,IBDV and HVTV vaccines ;respectively

Fig.2. Shows the results of Multiplex- PCR for detection of CAV as experimental contaminant in HVTV Vaccine; Lane M: 100 bp ladder,Each of lanes 1 & 2 ,show two fragments of 713 &388 bp representing CAV &HVTV respectively; Lane 3 ,show one fragment only of 388 bp of HVTV vaccine.Lane 4: negative control

Fig.3. Shows the results of Multiplex- PCR for detection of CAV as experimental contaminant in IBDV Vaccine; Lane M: 100 bp ladder, Each of lanes 1 & 2 ,show two fragments of 713 &480 bp representing CAV &IBDVv respectively; Lane 3 , show one fragment only of 480 bp of IBDV vaccine. Lane 4: negative control

Multiplex-PCR and its sensitivity in detection of CAV as an extraneous virus contaminant.

Fig.3 showed the Multiplex-PCR products specific for both IBDV and CAV of 480 bp and 713 bp respectively. The detection limit of the Multiplex-PCR for CAV was 10^3 $TCID_{50}$ in 5 µl of the nucleic acids of experimentally contaminated IBDV.

On the other hand the Multiplex-PCR products specific for both HVTV and CAV of 388 bp and 713 bp; respectively were detected. The detection limits of the multiplex- PCR for CAV was 10^3 $TCID_{50}$ in 5 µl of the DNA of experimentally contaminated HVTV.

DISCUSSION

PCR is recognized to be a valid alternative method for viral identification and extraneous agents testing in live attenuated viral poultry vaccines. Molecular tests can be more sensitive and have a higher level of discrimination than conventional approaches. In comparison, methods that use live animals present several disadvantages, for example, they take more time to complete and indirectly test for the presence of virus; viruses are not detected if the injected animals fail to develop an antibody response *(6).*

The designed Multiplex-PCR in this study has a dual function where as beside the identity of the live attenuated viral vaccines (IBDV and HVTV), detection of the experimental viral contaminant (CAV). In order to verify the sensitivity of the Multiplex-PCR to detect the extraneous virus contaminant, CAV was serially diluted in the two vaccines. The Multiplex-PCR detects CAV to a limit of 10^3 $TCID_{50}$/5µl of the sample. These results proved the sensitivity of the designed Multiplex-PCR. It has been showed that *(7,8)* sensitivity of PCR exceed or was equal to that of mouse antibody detection tests. In vaccine control, new comparative studies assessing the performance of PCR testing indicate that molecular tests detect viral contaminants in veterinary vaccines at a lower concentration than serological methods, *(9).*

In order to simplify the validation and standardization of the Multiplex-PCR to identify a live attenuated viral vaccine and to detect the extraneous viral contaminant in such vaccine the following recommendations could be beneficial:

Using of common PCR instruments and tools for nucleic acid extraction; Using of commercial reaction mixes "ready to go PCR" are used to simplify ordering, reagent storage and assay set up also the master mixes offer convenience, speed and consistency specially when operating in a quality management system under ISO 17025.

The keys to the successful outline use of any test are establishment of standard operating procedures (SOPs) and validation/ documentation systems. Also nucleic acid amplification techniques including the Multiplex-PCR should be performed and validated according to the requirements *(2).* Method of analysis 2.6.21. "Nucleic acid amplification techniques" and finally the test must be under go inter-laboratory validation. Once an assay is validated, it is often not cost-effective to revalidate with a new product.

Conclusion

Multiplex-PCR is a valid *in vitro* alternative method for identity and extraneous agent testing of poultry vaccines. The advantages of PCR assays for detection of a wide range of DNA and RNA viruses encourage us to design sets of Multiplex-PCR assays for identity and extraneous virus testing of all the poultry viral vaccines.

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الملخص العربى

استخدام تفاعل البلمرة المتسلسل والمتعدد فى تعريف والكشف على الملوثات الفيروسية للقاحات الدواجن الفيروسية الحية

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المعمل المركزى للرقابة على المستحضرات الحيوية البيطرية

فى إطار استحداث طرق معملية جديدة لتقيم جودة لقاحات الدواجن الحية, تم استخدام تفاعل البلمرة المتسلسل المتعدد لعمل اختبارى التعريف والكشف عن الملوثات الفيروسية -فى تفاعل واحد- للقاحى الجمبورو والميرك (عترة HVT) وذلك بعد أجراء تلوث تجريبى بقوى عيارية مختلفة من فيروس انيميا الطيور للقاحين محل الدراسة.

ولتثبيت هذه الطريقة تم استخدام لقاح الجمبور الحى المستضعف -الملوث مسبقاً بفيروس انيميا الطيور- كأحد اللقاحات الفيروسية ذات الحامض النووى الريبوسومى (RNA), وبعد إستخلاص الحامض النووى الريبوسومى والديوكسى ريبوسومى لهذا القاح تم تحويل الحامض النووى الريبوسومى لفيروس الجمبورو الى cDNA كخطوة اولى بواسطة تفاعل RT تلاه تفاعل البلمرة المتسلسل المتعدد بأستخدام زوجين من البريمرات المتخصصة, الأول لفيروس الجمبورو والثانى لفيروس انيما الطيور.

من ناحية أخرى تم استخدام لقاح الميرك (HVT) الملوث تجريبياً ايضا بفيروس انيميا الطيور لتثبيت نفس الطريقة بالنسبة للقاحات الفيروسية ذات الحامض النووى الديوكسى ريبوسومى بأستخدام زوجين من البريمرات الأول خاص بفيروس الميرك (HVT) والثانى لفيروس انيميا الطيور (CAV) وقد اثبتت النتائج أن تفاعل البلمرة المتسلسل المتعدد شديد التخصص والحساسية حيث أنه تم التعرف من خلاله على كل نوع لقاح على حدة بجانب الكشف عن فيروس انيميا الطيور كملوث فيروسى بكل لقاح من اللقاحين محل التجربة حتى عند القوة العيارية 10^3 $TCID_{50}/5\mu l$ من العينة.

هذه النتائج تؤكد كفاءة أستخدام تفاعل البلمرة المتسلسل المتعدد فى تقييم جودة لقاحات الدواجن الفيروسية الحية من حيث التعرف عليها والكشف على الملوثات الفيروسية بها.