In-Vitro Testing Of The Safety Of Marek's Disease Virus Vaccines

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ABSTRACT

The traditional methods for safety testing of Marek's disease (MD) virus vaccines is based on the clinical signs and pathological alteration that recorded within 120 days post vaccination of one day old susceptible specific pathogen free chicks. To provide a rapid, sensitive and simple means of evaluating MD vaccine for freedom of oncogenicity, we have used the polymerase chain reaction (PCR). The primers chosen to detect MDV sequences flank the 132 bp tandem repeat of the Bam H1-H fragment, whose PCR product is specific for serotype 1 MDV. The CVI 988 vaccine yielded amplified products of various size corresponding to the number of 132-bp repeat units, while virulent GA strain yielded a strong 434 bp PCR product. Multiplex PCR contained primers specific for serotype 1 and 3, yielded 388 bp fragment specific for herpes virus of turkey (HVT) in addition to the above result. The sensitivity of the multiplex PCR was studied by experimental contamination of both CVI 988 and HVT by 100 and 10 plaque forming unit (PFU) of GA strain. The detected GA strain at the two concentrations indicates the high sensitivity of the multiplex PCR. Using multiplex PCR was found to be simple, rapid and sensitive for testing of MD vaccines for safety.

INTRODUCTION

Marek's disease (MD) is a lymphomatous and neuropathic disease of domestic fowl *(1)*, occurs at 3-4 weeks of age or older and is most common between 12 and 30 weeks of age.

MD is prevented by vaccinating chickens *in-ovo* or at one day of age using live viral vaccines. HVT in either a cell-free (lyophilized) form, or a cell-associated (wet) form is most commonly used. Attenuated variants of serotype-1 strains of MDV are also used as vaccines. Serotype-2 strains are used, particularly in bivalent vaccines together with HVT (serotype 3). Serotypes 1 and 2 vaccines are only available in cell-associated form. Bivalent vaccines consisting of serotypes 1 and 3 or 2 and 3 *(2)*, or trivalent vaccines consisting of serotypes 1, 2 and 3 are used to combat the very virulent strains of MDV that are not effectively well controlled by the usual monovalent vaccines.

The MD is progressive in nature with a relatively long incubation period *(3)*. The vaccine virus/es should be non-pathogenic for chickens when inoculating ten times the field dose into 1-day-old specific pathogen free chickens of a strain susceptible to MD, this ensures by absence of gross lesions of MD withen the 120 days post vaccination ***(4,5)***.

The genomes of all the MDV serotypes have been sequenced ***(6-8)***. Polymerase chain reaction (PCR) have been described that allow differentiation of oncogenic and non-oncogenic strains of serotype 1, and differentiation of MDV vaccinal strains of serotypes 2 and 3 ***(9-13)***.

The imported MD vaccines are routinely submitted to our laboratory for testing its efficacy and safety. The objective of this study was evaluation of a multiplex PCR method for testing the oncogenicity of the MD vaccines, or detection of oncogenic MDV as an extraneous virus contaminating the MD vaccines or any live viral poultry vaccines.

MATERIAL AND METHODS

MDV strains

a. Vaccines

Three MDV vaccines were submitted to our laboratory for evaluation. The first vaccine contained CVI 988 strain *(14)* representing MDV-1, the second vaccine contained the FC126-HVT strain *(15)* representing MDV-3 (LAH Co.) and the third contained both CVI 988 and FC 126 strains (Intervet, Boxmeer).

b. An oncogenic GA strain of MDV-1

This was obtained from Intervet, Boxmeer Co., and used routinely in our laboratory as a positive control for evaluation of MDV vaccines: The CVI 988 and HVT vaccines were experimentally contaminated by addition of 10 or 100 PFU of cell associated GA strain of MDV to each vaccine.

Cultivation of MDV

The MDVs were grown in primary chicken embryo fibroblasts (CEFs) from 9-day-old specific pathogen free chicken embryos (Kom Osheim, Fayoum) at 37°C in 5% Co_2 atmosphere according to ***(16)***. The cultures were examined daily for development of cytopathic effect (CPE).

Chickens

Chickens originated from specific pathogen free eggs (Kom Osheim) were used for MD vaccine safety study by conventional method.

Safety testing by traditional methods

Two hundred fifty chicks at hatch were divided randomly into five groups each of 50 chicks. The first group was inoculated with ten doses of CVI 988 (about 32 x 10^3 pfu in 0.2 ml/bird) subcutaneously at the back of the neck. Second group received 25 x 10^3 pfu of HVT/bird. The third one received bivalent CVI 988 + HVT at concentration of 39 x 10^3 pfu/bird. The fourth group received 500 pfu of virulent GA / bird. The fifth group was left non-treated negative control.

All chick groups were maintained separately for 120 days except group 4 only 70 days. All birds that die during the test were autopsied for macroscopic lesions of MD. At the end of the experiment all birds were killed and necropsied for presence of MD gross lesions ***(17)***.

DNA isolation

Total DNA was isolated from virus infected CEFs, infected cells were maintained until extensive CPE was evident ***(18)***. Cells were scraped into cell culture media, pelleted, washed once with phosphate buffered saline, and incubated for 4 hours to over night at 39°C in 10 volumes of NES and proteinase K solution (0.15M NaCl, 0.1 M EDTA, 1% sodium dodecyl sulfate, proteinase K at 100 μg/ml). The DNA was extracted twice with phenol-chloroform-isoamyl alcohol (25:24:1) and twice with chloroform-isoamyl alcohol (24:1) before precipitation with ethanol. Isolated DNA was resuspended in TE (10 mM Tris pH 8.01 and 1 mM EDTA) to be utilized in PCR.

Oligonucleotide primers

The first primer set were selected to detect the 132 bp nucleotide repeat sequence located within the Bam H1-H fragment according to ***(19)*** and designated as A1 and A2, which were employed to differentiate between oncogenic and non-oncogenic strains of MDV-1 as shown in Table 1. The second primer set designated as B1 and B2 was employed to detect serotype 3 strains of MDV (HVT strains), Table 1 ***(11)***.

Table 1. Sequence of the synthetic oligonucleotide primers

Primer	Specificity	Sequence	Location	Fragment size
A1	Serotype 1 of MDV	TACTTCCTATATAGATTGAGACGT	65 bp 5` to the tandem 132 bp repeat	434 and 566
A2		GAGATCCTCGTAAGGTGTAATATA	105 bp down-stream	
B 1	Serotype 3 of MDV	CGCGTACTGCGCCTGACG	231-248	388
B2		CAACTTCGCTCTTGACG	618-602	

Polymerase chain reaction (PCR)

Three PCRs were performed ***(11,19)***. In the first PCR, 1 µg of DNA extract of the CVI 988 strain, GA strain, HVT strain, combined HVT + CVI 988, or experimentally contaminated CVI 988 and HVT vaccines was mixed in a reaction mixture of 50 µl contained 10 mM tris HCl (pH 8.5), 50 mM KCl, 1.5 mM $MgCl_2$ and 200 µM of each deoxynucleotide and 50 Taq polymerase (Fermentas Co.). The amplification reaction was performed in a DNA thermal cycler (T-Gradient thermal cycler, Biometra). Following an initial template melting step at 95°C for 3 minutes. The DNA was amplified during 30 cycles of 94°C for 1 minute, 55°C for 1 minute and 72°C for 1 minute. A final elongation at 72°C for 10 minutes. On the other hand, in the second PCR, DNA extracts of HVT vaccine, the combined HVT+CVI 988 vaccines, CVI 988 vaccine, or the experimentally contaminated CVI 988 and HVT vaccines were utilized in a multiplex PCR contained 2 primer sets A1, A2, B1 and B2. The reaction mixture of 50 µl contained the same constituent of the previous reaction except 100 pmol of each primer and 0.5 µM of each deoxynucleotide. The PCR cycles were also the same as the previous reaction. The third PCR resemble the first except the using of B1 and B2 primers alone at concentration of 50 pmol of each DNA from uninfected CEF was used as negative control in the 3 PCRs. The PCR products were visualized by electrophoresis in 1.5% agarose gel with ethidium bromide staining at 100 volts. A 100 bp ladder was used.

RESULTS

A. Traditional method

The three vaccines under test were safe by the conventional method of evaluation where neither of the vaccine strains induced MD lesion in 120 day trial in groups (1, 2, 3 and 5). The virulent GA strain used as positive control induced 100 % gross lesions at 70 days (47/47, gross lesion/total), confirming the high susceptibility of chicken to MD.

First PCR

No PCR product was obtained with the DNA of uninfected CEF. A strong PCR product of 434 bp was detected with the oncogenic GA strain and the GA contaminated HVT vaccine at high and lower concentration of GA strain, representing a dimmer of the 132 bp tandem repeat (Fig. 1). PCR products of equal intensity containing up to 9 repeats of equal intensity of the 132 bp repeat were obtained with the monovalent CVI 988 vaccine and bivalent HVT+CVI 988, while an additional strong 434 bp fragment were obtained with PCR pattern of the experimentally GA contaminated CVI 988 vaccine illustrating the presence of both oncogenic and attenuated MDV-1 in the same sample of both concentration of GA strain. No PCR product was obtained with the HVT vaccine (Fig. 1).

Second PCR

No PCR product was obtained with the DNA of uninfected CEF. A 388 bp fragment was obtained with the HVT vaccine. While, up to 9 repeats of equal intensity of the 132 bp repeat were obtained besides the 388 bp fragment with the combined HVT+CVI 988 vaccine. The 9 repeats were visualized also with the CVI 988 vaccine. The most valid result in this test is presence of the strong 434 bp fragment that represent the pathogenic GA strain in the PCR pattern of the GA contaminated HVT and CVI 988 vaccines at both concentrations of GA strain (Fig. 2).

The third PCR

No PCR products were obtained with DNA of uninfected CEF, nor CVI 988 vaccine. On the other hand, a 388 bp fragment was obtained with monovalent HVT vaccine and bivalent HVT+CVI 988 vaccine (Fig. 3).

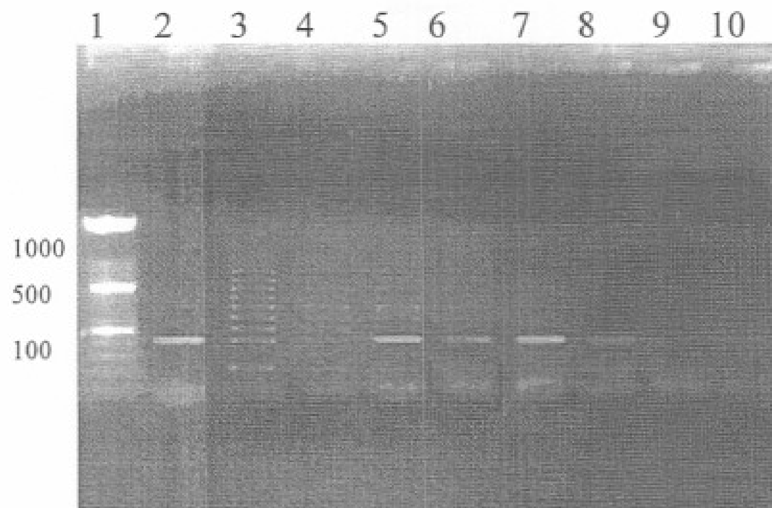

Fig. 1. First PCR

Lane 1: 100 bp DNA ladder
Lane 2: GA MDV strain
Lane 3: CVI 988 vaccine
Lane 4: CVI 988 + HVT vaccine
Lane 5: Experimentally contaminated CVI 988 vaccine with 100 pfu of GA strain of MDV
Lane 6: Experimentally contaminated CVI 988 vaccine with 10 pfu of GA strain of MDV
Lane 7: Experimentally contaminated HVT vaccine with 100 pfu of GA strain of MDV
Lane 8: Experimentally contaminated HVT vaccine with 10 pfu of GA strain of MDV
Lane 9: HVT vaccine
Lane 10: Non-infected CEF cells

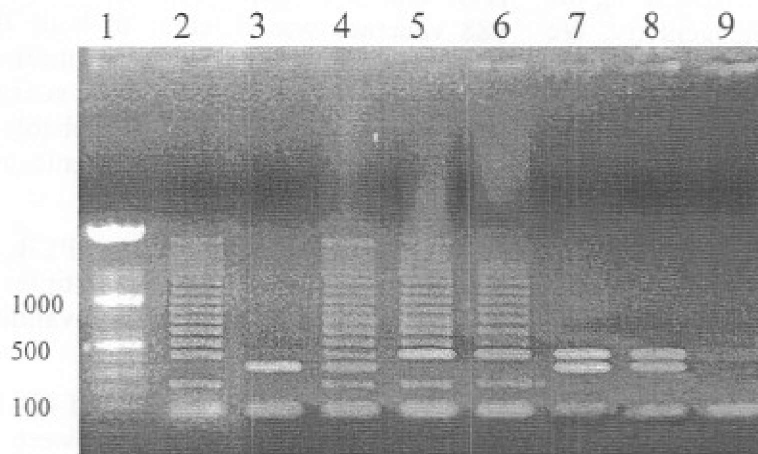

Fig. 2. Multiplex PCR

Lane 1: 100 bp DNA ladder
Lane 2: CVI 988 vaccine
Lane 3: HVT vaccine
Lane 4: CVI 988 + HVT vaccine
Lane 5: Experimentally contaminated CVI 988 vaccine with 100 pfu of GA strain of MDV
Lane 6: Experimentally contaminated CVI 988 vaccine with 10 pfu of GA strain of MDV
Lane 7: Experimentally contaminated HVT vaccine with 100 pfu of GA strain of MDV
Lane 8: Experimentally contaminated HVT vaccine with 10 pfu of GA strain of MDV
Lane 9: Non-infected CEF cells

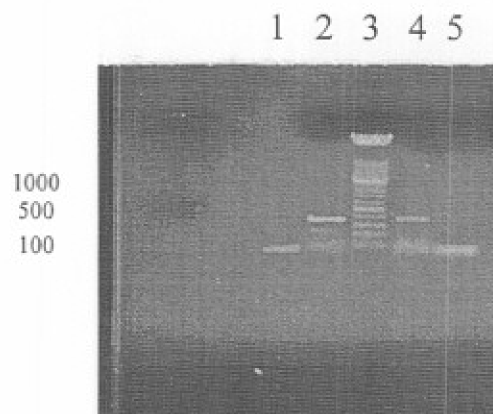

Fig. 3. Third PCR

Lane 1: Non-infected CEFs
Lane 2: HVT vaccine
Lane 3: 100 bp DNA ladder
Lane 4: CVI 988 + HVT vaccine
Lane 5: Non-infected CEF cells

DISCUSSION

In our study, we try to provide rapid and sensitive means of testing the safety of Marek's disease virus vaccines by assuring the presence or absence of the oncogenicity. We have applied 3 PCRs for this purpose. For primary assessment of the used primers, an oncogenic GA strain of MDV, a non-oncogenic Rispense vaccine (CI 988 strain), which represent serotype 1 and HVT vaccine (FC 126 strain), which represent serotype 3 were used.

The safety of three vaccines under test were approved by the results of the traditional methods ***(4,17)***.

The first PCR for MDV is designed to detect only serotype 1 MDV and the product size is indicative of oncogenic or attenuated MDVs. The amplified region is the 132 bp tandem repeat sequence which is located in the Bam H1-H fragment (***9,10,11,20,21***).

MDV attenuation is accompanied by expansion of the 132 bp segment in the repeat long region *(22-24)*.

The result of the 1st PCR confirm the above findings, the oncogenic GA strain produced a strong 434 bp fragment representing a dimmer of the 132 bp repeat tandem, while the CVI 988 vaccine strain produced 9 fragments of the 132 bp repeats. The same results were previously obtained ***(19)***. The GA contaminant of HVT and CVI 988 vaccines was detected at both 100 PFU and 10 PFU levels of GA concentrations. This result is indicative for the high sensitivity of PCR. One infected cell was probably enough to differentiate between oncogenic and non-oncogenic MDVs ***(11)***.

The result of the second PCR, where 2 primer sets were used (Multiplex PCR), confirm the sensitivity and high validity of the multiplex PCR.

The same PCR product of 434 bp and 9 fragment of the 132 bp repeats were obtained with GA strain and CVI 988 strain, respectively, besides the 388 bp with the HVT strain. The most valid and higher important result of this test is the detection of the GA contaminant in the experimentally contaminant HVT and CVI 988 vaccines at either 100 PFU or 10 PFU level of GA strain.

The 3rd PCR were designed for detection of HVT strains and also for confirmation of the sensitivity of the established multiplex PCR. The PCR product

of the HVT vaccinal strain have fragment of 388 bp length. These consistent with that previously reported ***(11)***.

In conclusion, the PCR results were reliable and direct method to detect the oncogenicity of MD vaccinal strain, saving time (results can be obtained in less than 48 hours) simple, easy to perform, highly sensitive and avoiding problematic effect of susceptibility of chicken to MD used in testing the efficacy and safety of imported MD vaccines.

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الملخص العربى

اجراء اختبار الآمان للقاحات المضادة لمرض الميرك معمليا

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المعمل المركزى للرقابة على المستحضرات الحيويه البيطريه – مركز البحوث الزراعية

طبقاً لبروتوكولات معايرة لقاحات الميرك الموصى بها من قبل المكتب العالمى للأوبئة OIE فإن اختبار الآمان الخاص بلقاحات الميرك يتم اجراؤه بحقن كتاكيت عمر يوم – خالية من المسببات المرضية وقابلة للإصابة بالميرك – باللقاح محل الدراسة فى مقابل مجموعة أخرى من نفس مصدر الكتاكيت يتم حقنها بعترة مسرطنة من فيرس الميرك ومجموعة أخرى لا تحقن وتستخدم كمجموعة ضبط سالبة . وتستمر ملاحظة هذه المجموعات الثلاث لمدة سبعون يوماً حيث يتم ذبح دجاج المجموعة الثانية للتأكد من وجود أورام سرطانية واصابات بالميرك ومن ثم تستكمل التجربة حتى عمر مائة وعشرون يوماً حيث تذبح المجموعة الأولى للتأكد من عدم وجود أورام سرطانية بها وبذلك نؤكد أن اللقاح محل الاختبار آمن. ولتلافى سلبيات الاختبار السابق ولتوفير الوقت والجهد والتكلفة قمنا بهذه الدراسة أو المحاولة حيث استخدمنا تفاعلى Multiplex RCR,PCR وباستخدام نوعين مختلفين من ال Primers :النوع الأول للكشف عن عترة فيرس الميرك رقم ١ وأيضاً للتفرقة بين السلالات الضعيفة والمسرطنة من هذه العترة من فيرس الميرك أما النوع الثانى من ال Primers فاللكشف عن عترة فيرس الميرك رقم ٣(المعزولة من الرومى HVT) ولقد صممت هذه الدراسة للتأكد من دقة وتخصص وحساسية اختبار ى ال PCR ، Multiplex PCR محل الدراسة ، فتم اختبار ثلاث لقاحات ميرك الأول يمثل العترةالأولى (Rispense) والثانى يمثل العترة الثالثة (HVT) والثالث يمثل لقاح مزدوج من العترتين١ ، ٣ ثم قمنا بعمل تلوث تجريبى لعدد (٢) امبولة من كل لقاح . الأولى باستخدام عترة (GA)المسرطنة من فيرس الميرك بتركيز 100 PFU والثانية بتركيز 10PFU وقد تم اجراء استخلاص الحامض النووى DNA لكل العينات محل الدراسة واجراء اختبارى ال PCR ، Multiplex PCR لها وكانت النتائج مبهرة حيث أمكن التفريق بين

كل عترة من لقاح الميرك ، وأيضاً اللقاح المزدوج والأهم – فقد أمكن اكتشاف وجود الملوث الفيروسى المسرطن من فيرس الميرك حتى فى تركيزه الأقل .