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Journal of Virological Methods 193 (2013) 508–511

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

The use of RT-PCR for determination of separate end-points for the strains IB H120 and IB D274 in titration of the combination vaccine Poulvac IB® primer

H.J. Geerligs[a,*], C.A.M. Meinders[b], J. Snel[b], W. Duyves[b]

[a] Zoetis, VMRD, Mercuriusstraat 20, 1930 Zaventem, Belgium
[b] PGM Pfizer Animal Health, Van Houtenlaan 36, 1381 CP Weesp, The Netherlands

Article history:
Received 1 October 2012
Received in revised form 12 June 2013
Accepted 21 June 2013
Available online 1 July 2013

Keywords:
Titration
Poultry
Eggs
Live combination vaccine
PCR
End-point

A B S T R A C T

Poulvac IB® Primer is a lyophilized vaccine containing two attenuated infectious bronchitis strains in one vial, IB H120 and IB D274. For quantification of the viral content of the vaccine, dilution series of the final product are inoculated in embryonated chicken eggs. After the incubation period of seven days standard practice is for the embryos to be taken from each egg and examined visually for IB specific lesions; these readings are used to determine an end-point in viral titrations. The result is a titre value to which both strains contribute. However, it is not clear what the live virus titre is for strain IB H120 and for strain IB D274. In order to determine end-points in the titration for each of the two strains, we collected the allantoic fluids from each egg after the incubation period and tested these for the presence of IB H120 and IB D274 by a strain specific reverse phase PCR. Based on the data obtained by PCR we were able to determine an end-point for each of the two strains. For a given commercial batch of Poulvac IB primer we determined titres of $10^{6.31}$ EID_{50} per vial for IB H120 and $10^{6.59}$ EID_{50} for IB D274 using PCR for end-point determination. These end-points matched well with the end-point determined for both strains cumulatively after visual examination, i.e. $10^{6.67}$ EID_{50} per vial. It is concluded that PCR is a suitable means to determine end-points in titrations of live viruses.

1. Introduction

Infectious bronchitis virus (IBV), a coronavirus, is one of the foremost causes of economic losses within the poultry industry, affecting the performance of both meat-providing and egg-laying chickens (Cavanagh, 2007; De Wit et al., 2011). The major reason for the high profile of IBV is due to the existence of a very large number of serotypes, although the virus is also highly contagious. In order to control the disease in poultry, both live and inactivated IB vaccines are used extensively. The nature of the protective immune response to IBV is poorly understood, but research has demonstrated that the surface spike protein, and particularly the amino-terminal S1 portion, is sufficient to induce good protective immunity. There is increasing evidence that only a few amino acid differences amongst S proteins are sufficient to have a detrimental impact on cross-protection (Cavanagh, 2007). As it is not always clear which serotype is responsible for any health problems in poultry, combinations of vaccines are widely used to generate broad protection (Terregino et al., 2008). Another possibility is to use vaccines which contain a combination of different vaccine viral strains. Zoetis is marketing a live combination vaccine for chickens named Poulvac® IB Primer. This lyophilized vaccine contains two attenuated infectious bronchitis strains, IB H120 (MacDonald and McMartin, 1976) and IB D274 (Kusters et al., 1987). Both vaccine strains have been attenuated by passage in embryonated chicken eggs, which is the usual method for attenuation of infectious bronchitis viruses and development of vaccines (MacDonald and McMartin, 1976; Klieve and Cummings, 1988; Jackwood et al., 2003; Liu et al., 2007). In order to quantify the viral content of the vaccine, dilution series of the final product are inoculated in 10 days embryonated chicken eggs. After the incubation period the eggs are opened and the embryos removed for examination of IB specific lesions. The product titre defined is the reciprocal value of the dilution causing embryonal lesions in 50% of the eggs. For the combination vaccine Poulvac® IB Primer the result is a cumulative titre to which both IB strains contribute. In order to titrate both virus strains separately, strain specific neutralizing antisera have been tested. It has been shown that antiserum against strain IB D274 does not cross-react with strain IB H120 (Kusters et al., 1987) and it was speculated that antiserum against IB H120 would not cross react with strain IB D274. However, during titration experiments it was determined

* Corresponding author. Tel.: +32 2 7157593; fax: +32 2 4012407.
E-mail addresses: harm.geerligs@zoetis.com, harm.geerligs@pfizer.com (H.J. Geerligs).

0166-0934/$ – see front matter © 2013 Published by Elsevier B.V.
http://dx.doi.org/10.1016/j.jviromet.2013.06.029

Table 1
PCR primers for detection of the strains IB H120 and IB D274, with the respective PCR product size in base pairs.

IB strain	Sequence 5′-3′ forward primer	Sequence 5′-3′ reverse primer	Length in base pairs
IB D274	5′-ATACAATTATATCAAACCAGC-3′	5′-AATACAGATTGCTTACAACCACC-3′	211
IB H120	5′-AATACTACTTTTACGTTACAC-3′	5′-AATACAGATTGCTTACAACCACC-3′	299

that the antiserum against IB H120 strongly reacted with IB D274. PCR has been used successfully to detect IB strains in various samples and it appeared to be possible to distinguish IB strains, including IB H120 and IB D274, based on their reactivity with primers specific for the various strains (Worthington et al., 2008). Here, we took advantages of the genomic level differences between the two IB strains and describe a PCR based approach to quantify the titres of each IB virus strain contained in the Poulvac IB vaccine.

2. Materials and methods

2.1. Vaccines

Lyophilized vials of a routine production batch of Poulvac® IB primer were used. During the manufacturing process 4 ml of a mixture containing allantoic fluid with IB H120, allantoic fluid with IB D274 and a freeze-dry stabilizer was filled per vial. The vials were lyophilized according to routine procedures and subsequently stored in the dark at 5 ± 3 °C.

2.2. Titration in embryonated specific pathogen free chicken (SPF) eggs

Titrations of IB virus were performed according to standard procedures as described by Doherty (1967). Specific pathogen free chicken (SPF) eggs were obtained from ValoBioMedia, Osterholz-Scharmbeck, Germany. Three vials of each vaccine batch were tested per titration assay. The content of each vial of lyophilized product was reconstituted in 4 ml water for injection. Each sample was diluted further in nutrient broth (Becton Dickinson, Breda, The Netherlands) containing 50 μg/ml gentamicin sulphate (Sigma–Aldrich Chemie BV, Zwijndrecht, The Netherlands), up to the beginning of the dilution series to be inoculated in eggs. In this series 10-fold dilution steps were made and per dilution 6 10 days embryonated eggs were inoculated. After inoculation the eggs were incubated at 37 °C and a relative humidity of 55%. After 1 day incubation eggs with dead embryos were considered nonspecific deaths and discarded. After an incubation period of 7 days the eggs were opened and the allantoic fluid harvested and stored at −70 °C. The embryos were removed and examined for the presence of specific lesions caused by the virus, i.e. dwarfing, curling and stunting of the embryo. Dead embryos were considered positive for IBV. The titre, expressed as the number of EID_{50}/ml was calculated according to the method of Spearmann-Karber (Finney, 1964).

2.3. Purification of RNA

Allantoic fluids from the eggs were investigated for the presence of IB virus by reverse transcriptase PCR with strains specific reverse transcriptase PCR primers for IB H120 and IB D274. The RNA was isolated from each allantoic fluid samples using a High Pure Viral RNA kit (Roche, Almere, The Netherlands) according to the instructions of the manufacturer.

2.4. PCR analysis

For PCR analysis a PCR master mixture was prepared. For each sample to be analysed 2.0 μl working solution forward primer (100 μM primer), 2.0 μl working solution reverse primer (100 μM primer), 1.0 μl Taq-mix, 25.0 μl 2× buffer, was mixed with 15.0 μl PCR grade water. Taq-mix and 2× buffer had been obtained from the Super script One Step Reverse Transcriptase PCR Kit (Invitrogen, Bleiswijk, The Netherlands). For one sample 45 μl of this mixture was added to a PCR tube containing 5.0 μl of the RNA sample. 5.0 μl of PCR grade water was used instead in case of a negative control. The primers for the strains IB H120 and IB D274 and the size of the PCR products are indicated in Table 1. The tubes were placed in the PCR machine (Thermocycler, MJ Research PTC-200, Bio-Rad, Veenendaal, The Netherlands) and the following steps were programmed: cDNA synthesis 30 min at 50 °C, initial denaturation 10 min at 95 °C, 30 cycles of 30 s at 95 °C, 30 s at 50 °C, 45 s at 72 °C. Final extension was 7 min at 72 °C and reaction stop 5 min at 4 °C. The formation of PCR product was assessed by agarose gel electrophoresis. First the gel was prepared. TBE buffer was prepared by 10-fold dilution of a solution with 1.0 M Tris, 0.9 M boric acid, 0.01 M EDTA in water. Agarose was added to a concentration of 2% to the TBE buffer and ethydium bromide to a final concentration of 0.5 μg per ml. The gel was allowed to solidify and used for electrophoresis of the PCR samples. The samples were allowed to go through the gel for 2 h at 70 V. A basepair ladder was used to determine the size of the bands in the agarose gel. Gel electrophoresis was stopped when separation of the different bands in the base pair ladder was satisfactory. Bands were made visible by ultraviolet illumination.

3. Results

In a first experiment, a dilution series of a batch of Poulvac® IB primer was prepared according to routine production QC procedures. The dilution series ranged from 10^{-4} to 10^{-9}. All dilutions were tested for presence of IB H120 and IB D274 by reverse transciptase PCR. None of the dilutions was positive for IB by PCR, see Table 2. Apparently, the quantities of virus in the dilutions series were below the detection level.

The dilution series was inoculated in embryonated SPF chicken eggs, 6 eggs per dilution. After the routine incubation period samples of the allantoic fluids were tested also by PCR. It now appeared that after the egg passage dilutions up to 10^{-6} were positive by PCR for IB D274 and dilutions up to 10^{-7} were positive by PCR for IB H120, see Table 2. The embryos were also examined visually, and the results show that at a dilution of 10^{-6} all (5 of 5) embryos were affected by the virus. At a dilution of 10^{-7} one of 5 embryos was affected. The only possible explanation for the presence of IB H120 and IB D274 in the allantoic fluids was that the viruses had been able to cause a productive infection in the eggs. It is concluded that in this test live virus titres were determined.

In a second experiment a routine titration on Poulvac® IB primer was performed. This titration was performed in triplicate. After the incubation period the eggs were opened and the embryos were examined visually. Of each triplicate a titre per vial was calculated (see bottom Table 3 under Visual) and from the 3 scores obtained, i.e. 6.83, 6.37 and 6.80 $^{10}\log EID_{50}$ respectively, an average titre per vial was calculated, i.e. 6.67 $^{10}\log EID_{50}$ or $10^{6.67}$ EID_{50}. Analysis of allantoic fluids demonstrated for both strains there were clear endpoints in each triplicate so that a titre could be determined in a correct manner. The average titres determined for both strains were $10^{6.31}$ EID_{50} per vial for IB H120 and $10^{6.59}$ EID_{50} for IB D274. If the sum of these 2 values is determined the titre is $10^{6.77}$ EID_{50}

Table 2
Titration of Poulvac IB primer vaccine using strain specific PCR for detection of IB H120 or IB D174. Results from a dilution series before titration and allantoic fluid pools after titration, and results of visual examination after titration are shown.

Dilution	PCR on dilution series		Results from egg titration		
	IB H120	IB D274	Embryos affected by IB visually	PCR on pooled allantoic fluids	
				IB H120	IB D274
10^{-6}	–	–	5 of 5	+	+
10^{-7}	–	–	1 of 5	+	+
10^{-8}	–	–	0 of 5	+	–
10^{-9}	–	–	0 of 5	–	–

per vial, which is very near to the value of $10^{6.67}$ EID_{50} obtained after visual examination.

4. Discussion

Numerous publications are available in which viral titrations and possible issues have been described. For several viruses tissue culture methods can be used, in which a positive response can be observed by examination of monolayers of cells for the presence of a cytopathogenic effect (Heldt et al., 2006). If the virus does not form a cytopathogenic effect, the presence of live virus in the culture can be demonstrated by methods using colour agents (Heldt et al., 2006) or specific antibodies (Zielinska et al., 2005; Yap and Lam, 1994). For avian viruses suitable substrates are needed and sometimes there are different possibilities (Nicholas et al., 1986). For titrations in embryonated eggs the presence of the virus in allantoic fluid can be tested using a specific property of the virus, for example its ability to cause haemagglutination (Klimov et al., 2012). The presence or absence of lesions on the embryos also can be used to determine the presence of live virus, such as has been demonstrated for IB viruses (De Wit, 2000). The use of PCR for screening samples from titrations for the presence of virus has not been described previously. Initially PCR was used mainly for testing for the presence of viruses or other infectious agents in samples (Tafuro et al., 1996). Currently, with the introduction of real time PCR it is possible to determine quantities of virus particles in samples (Hiroshi Kimura et al., 1999; Isabel Costafreda et al., 2006; Spackman and Suarez, 2008). Because of its specificity it is possible also to detect more than one virus strain in one test using PCR (Van Elden et al., 2001).

We used PCR for detection of two different IB virus strains in the allantoic fluids from all the individual eggs in a titration of a live IB combination vaccine. Based on the results of the PCR we determined whether an allantoic fluid was positive for one or both of the viruses or not, and as such we were able to define a separate

Table 3
Titration test performed in triplicate with visual examination of embryos and testing of allantoic fluids from each egg by strain specific PCR.

Dilution	Visual	PCR		Dilution	Visual	PCR		Dilution	Visual	PCR	
		D274	H120			D274	H120			D274	H120
10^{-4}	+	+	+	10^{-4}	+	+	+	10^{-4}	+	+	+
	+	+	+		+	+	+		+	+	+
	+	+	+		+	+	+		+	+	+
	+	+	+		+	+	+		+	+	+
	+	+	+		+	0[b]	0		+	+	+
	+	+	+		+	+	–		+	+	+
10^{-5}	0[a]	0	0	10^{-5}	+	–	+	10^{-5}	+	+	+
	+	+	+		+	+	+		+	+	+
	+	+	+		+	+	+		+	+	+
	+	+	–		+	+	+		+	+	+
	–	–	–		+	+	+		+	+	+
	+	+	+		+	+	+		+	+	+
10^{-6}	+	–	+	10^{-6}	+	–	+	10^{-6}	0[a]	0	0
	+	–	+		–[c]	–	+		+	+	–
	–	–	–		–	–	–		–	–	–
	–	–	–		–	–	–		–	–	–
	+	+	+		–	–	–		+	–	+
	–[c]	+	+		–	–	–		+	–	+
10^{-7}	+	–	+	10^{-7}	–	–	–	10^{-7}	–	–	–
	+	+	–		–	–	–		–	–	–
	–	–	–		–	–	–		–	–	–
	–	–	–		–	–	–		–	–	–
	–	–	–		–	–	–		–	–	–
	–	–	–		–[c]	–	+		–	–	–
10^{-8}	–	–	–	10^{-8}	–	–	–	10^{-8}	–	–	–
	–	–	–		–	–	–		–	–	–
	–	–	–		–	–	–		–	–	–
	–	–	–		–	–	–		–	–	–
	–	–	–		–	–	–		–	–	–
	–	–	–		–	–	–		–	–	–
Titre	6.83	6.50	6.63	Titre	6.37	6.03	6.53	Titre	6.80	6.40	6.60

[a] Egg lost within 24 h after inoculation.
[b] No PCR results because of problem with RNA isolation.
[c] Difference in result between visual examination and PCR.

endpoint in the live virus titration for each of the two strains. It has to be clear that this is not the same as a quantitative PCR. In a quantitative PCR the number of genomic fragments is determined based on the number of cycles in the real time PCR required for a positive response. In such a PCR genomic fragments coming from live as well as 'dead' virus particles are determined. In the test described in this paper we determined whether an egg was positive for the presence of virus coming from a productive infection using PCR with a fixed number of cycles. Only a live virus particle can cause a productive infection. If the egg would have been inoculated with a dead virus particle, it would not have given a positive response in the PCR.

The titration method using PCR for testing the allantoic fluids is highly suitable to determine titres of different vaccine strains in one presentation. The PCR could be used also for end-point determinations of other IB strains, for example IB strains which do not have a very clear effect on embryos making it difficult to read egg titrations visually. Furthermore, the use of PCR for end-point determination makes it possible also to perform titrations on a large scale using specialized equipment.

5. Conclusion

It was demonstrated that PCR is a suitable means to determine end-points in titrations of live viruses. Further research will be required to investigate repeatability and reproducibility of these methods for virus titrations.

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