Using Polymerase Chain Reaction for Detection of Mycoplasma Spp. Contaminant in Live Attenuated Poultry Viral Vaccines

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Abstract: This work was done to assess the accuracy and performance of polymerase chain reaction (PCR) as a test for detection of mycoplasmal contamination of live attenuated viral poultry vaccines in comparison with the traditional method (culturing and microscopical examination). For this purpose experimental contamination of 10 different Newcastle disease vaccines batches from 10 different companies was done by adding 1ml of mycoplasmal broth culture containing $5x10^8$ CFU/ml of the reconstituted vaccines. Using traditional method, six vaccines showed positive results for mycoplasmal contamination. Ten vials from one of positive vaccines were experimentally contaminated with mycoplasma by same way after serial dilution, then examined using both the traditional method and PCR. Mycoplasmal contamination was detected up to dilution of 10^{-7} by both methods. One of negative vaccines was diluted 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} then contaminated with mycoplasma, after addition of pig serum to the medium as a source of cholesterol which neutralize the effect of inhibitors. PCR was able to detect mycoplasma contamination with dilutions of 10^{-3}, 10^{-4} and $10^{-5.}$ It could be concluded the PCR is most robust testing for detection of mycoplasma even those difficult to isolate in culture and saving time and effort.
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1. Introduction

Viral vaccines are generally produced in cell lines of diploid cell strains (MRC-5), chick fibroblast, embryonated eggs, continuous cell lines (Vero) and occasionally live animals. Contamination of cell lines by Mycoplasma is a well-documented phenomenon, large scale surveys revealing contamination rates of 5 to 50% (**Thornton, 1986**). The source of contamination may arise from media, sera or reagents contaminated with Mycoplasma and persons is main source of contamination (**Farzaneh and Laleh, 2012**). Mycoplasma, the smallest known free living organisms, are relatively common bacterial contaminant of mammalian cell cultures, Mycoplasma present particular challenges because they are difficult to detect using traditional microbiological techniques (**Livak, 1995**). The diagnosis of avian mycoplasma is mainly based on culture and serology, but culture is long and fasteious Mycoplasma isolation can suffer from contamination by fast growing microoorganisms. The most used serological test is rapid slide agglutination lacks of specifity. For all these reasons some veterinary diagnostic laboratories have developed molecular methods for detection of avian mycoplasma (**Spaepen, 1992**).

The present work aims to determine a suitable, accurate, rapid test to detect mycoplasma contamination in veterinary vaccines. The target test is PCR using a universal primer to reduce the time and effort required for mycoplasma determination.

2. Material and Methods

1- Vaccines:

Samples of ten live attenuated Newcastle disease (ND) commercial vaccines representing l0 different batches and different companies were used in this study.

2-Mollicute strain:-

Reference strain of *Mycoplasma gallisepticum* was kindly supplied by the Strain Bank Department in Central Laboratory for Evaluation of Veterinary Biologics, Abbasia, Cairo (CLEVB).

3-Oligonucleotide primer(Biomatik):-

Universal primer for identification of Mycoplasma spp. (**McAuliffe et al., 2005**):
GC341F(5'CGCCCGCCGCGCGCGGCGGGCGGG GCGGGGGCACGGGGGGCCTACGGGAGGCAG CAG-3')R53 (5'-ACCTATTATTACCGCG-3).The primer pair will be used at concentration of 20 pmol/reaction for each one. The expected amplicon will be 340 bp regain of16S rRNA gene of Mycoplasma genus.

4-DNA extraction:-

DNA was extracted by using DNeasy Blood and Tissue Qiagen following the manufacturer's instructions.

5- Pig serum (Biowest):-

Pig serum was added to Mycoplasma (PPLO)

media to neutralize the effect of Mycoplasma inhibiting materials that could be present in some vaccines under test according to method of **Paul and Carl (1965).**

6-Experimental design:-

The 10 examined vaccines were experimentally contaminated with lml broth of Mycoplasma gallisepicum stock strain adjusted to contain 5×10^8 CFU/ml, then examined for detection of Mycoplasma contamination according to **OIE (2013)**.

Positive samples will considered free from inhibitors and Vice versa for negative samples. At this point one vaccine that considered contaminated will be used to compare between the sensitivity and specifity of the PCR against the conventional method. On the other hand, one vaccine that considering containing Mycoplasma inhibitors will be used for the same purpose in addition to neutralize the inhibitor effect which prevent mycoplasma detection.

Fulfill these objectives trials were applied as following:

Trial (1):

Ten live attenuated NDV vaccines were reconstituted in 30 ml PPLO broth and experimentally contaminated with 1ml of mycoplasma stock and incubated at 37°C for 3 days in Co_2 incubator, then inoculated into Mycoplasma (PPLO) agar and examined for detection of mycoplasma contamination microscopically (traditional method).

Trial (2):

10 vials from one of the vaccines that gave positive result in trial (1) were contaminated artificially with different dilutions of serially diluted mycoplasmal broth starting from 10^{-1} ending with 10^{-10} and incubated at 37°C for 3 days at Co_2 incubator then cultivated on PPLO agar and examined for presence of mycoplasmal contamination by ordinary method using microscope and by PCR.

Trial (3)

One of the considered negative vaccines in trial (1) was reconstituted then diluted in broth from dilution10^{-1} till 10^{-5} and infected with *Mycoplasma gallisepticum* stock then the mycoplasmal contamination was detected using PCR,versus to the traditional method.

Trial (4)

In a trial to improve the traditional method one vial of same negative vaccine that used in trial (3) was reconstituted, then diluted from10^{-1} till 10^{-5} with adding cholesterol (pig serum) to medium for neutralization of the effect of inhibitors which may be present in the vaccines then contaminated with *Mycoplasma gallisepticum* stock and examined for presence of the mycoplasma contamination by traditional method (Microscopically).

3. Results and Discussion

Mycoplasma is the smallest free living organism that contained only by cell membrane without a rigid cell wall, Detection of Mycoplasma contamination using traditional method (Microscopically) has two main disadvantages duo to long duration and non cultivable stains (**Yuan et al., 2003**).

In trial (1) mycoplasmal contamination was detected in 6 vaccines out of the ten examined vaccines while the contamination couldn't be detected in 4 vaccines by traditional cultural method.

On trial (2) both the traditional and PCR methods could detect mycoplasma till the dilution of 10^{-7} (photo 1).

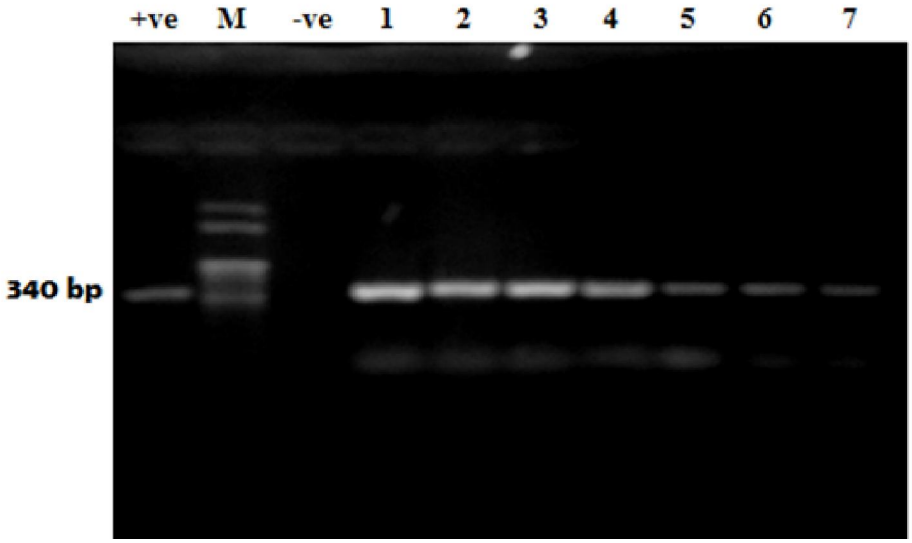

Photo (1): shows PCR product of one of diluted positive vaccines contaminated with Mycoplasma and detected till dilution 10^{-7}

In trial (3) PCR could detect mycoplasma contamination in all dilutions of the vaccine (photo 2) in agreement with **Fanrong et al. (2001).** While traditional method could not detect mycoplasma contamination before the dilution 10^{-4} , this finding was similar to that obtained by **Butler and Knight (1960)**.

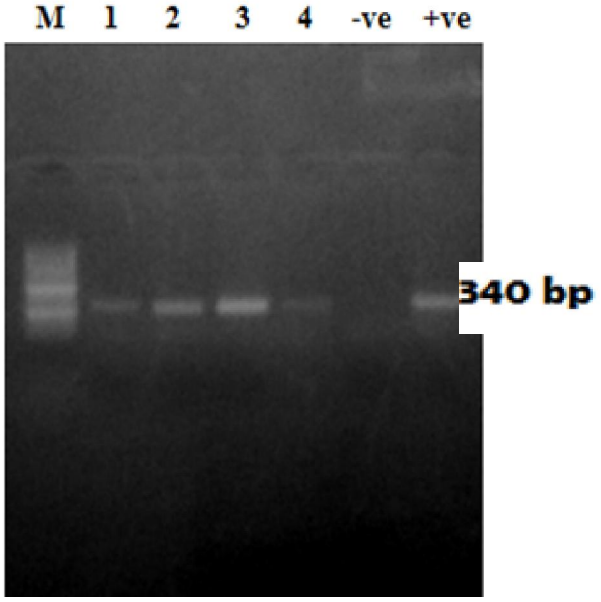

Photo (2): shows PCR product of one of diluted negative vaccines contaminated with Mycoplasma and detected from dilution 10^{-1}

In trial (4) and after adding of pig serum to the medium as a source of cholesterol, traditional method could detect mycoplasma contamination from dilution 10^{-3} till 10^{-5} in agreement with **Paul and Carl (1965)**.

These results indicates that PCR is highly sensitive than traditional method which is suitable only for cultivable species of mycoplasma and exhausts a long time (**Yuan et al., 2003**). It was important to find other method for detection of mycoplasma contamination, adding cholesterol improved the results somewhat but couldn't solve the main two problems concerning the non cultivable strains of mycoplasma and long time, while using PCR for detection of mycoplasma contamination saved time and was suitable for non-cultivable strains (**Razin, 1998**).

So from previous data PCR could be considered the most robust testing for mycoplasma contamination detection in poultry vaccines because it can detect most common moll cute species of mycoplasma even those hard to isolated in culture and saves time.

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