Detection of Mycoplasma DNA in Veterinary Live Virus Vaccines by the Polymerase Chain Reaction

Akemi KOJIMA, Toshio TAKAHASHI, Mayumi KIYIMA, Yasuaki OGIKUBO, Yutaka TAMURA, and Ryo HARASAWA

National Veterinary Assay Laboratory, 1–15–1 Tokara, Kokubunji, Tokyo 185, 1Animal Center for Biomedical Research and Branch Hospital, Faculty of Medicine, The University of Tokyo, Bunkyo-ku, Tokyo 113, Japan
(Received 7 July 1995/Accepted 4 June 1996)

Abstract. We examined bovine, porcine, canine, feline, avian and mink veterinary live vaccines for the presence of mycoplasma DNA by the polymerase chain reaction including nested-pair primers. Mycoplasma DNA was detected in 22 of the 61 commercial veterinary vaccines (36.1%), although these vaccines did not contain any viable mycoplasma cells. — Key words: mycoplasma contamination, polymerase chain reaction, vaccine.


At the national assay in Japan, the current test for the detection of mycoplasma contamination has been conducted on avian live virus vaccines, mainly prepared from chick embryo cell cultures and embryonated eggs, by a direct culture method according to the minimum requirement [10]. Two avian mycoplasma species, M. gallisepticum and M. synoviae, have been generally recognized as severe pathogens to domestic fowls. Therefore, mycoplasma contamination tests at the national assay are particularly essential for avian live virus vaccines.

Recently, the contamination of mycoplasmas in cell cultures, serum, and lyophilized viral strains have been reported [1, 2, 4, 5]. This is a severe problem for the quality control of veterinary vaccines because these biological materials are frequently used in vaccine production. In fact, there have been some reports of mycoplasma detection in live virus vaccines [3, 6, 8, 11, 13, 16]. However, in Japan, little is known about the situation of mycoplasma contamination in veterinary live virus vaccines except for the avian live virus vaccine conducted at the national assay.

The polymerase chain reaction (PCR), an in vitro DNA amplification method, allows the detection of a variety of microorganisms [9, 12, 15], and several attempts to detect mycoplasma contamination in cell cultures by PCR have been reported [4, 5]. Furthermore, enzymatic detection by PCR (ED-PCR) for the rapid detection of mycoplasma in cell cultures has been developed [7], and it was determined by agarose gel electrophoresis that ED-PCR products were amplified to an approximately 160 bp long segment. We confirmed that ED-PCR can react with many kinds of human and animal originated mycoplasma, for example, M. gallisepticum, M. synoviae, M. iners, M. glyophilum, M. pulorum, M. gallinarum, M. lipoaciens, M. iowae, M. orale, M. arginini, M. californicum, M. flocculare, M. sarlvi and M. hypopneumoniae, but does not react with Escherichia coli, Haemophilus paragallinarum, Salmonella typhimurium, S. gallinarum-pulorum, S. enteritidis, Staphylococcus aureus and Clostridium perfringens (data not shown). The detection limit of ED-PCR was less than 100 cfu per assay for these species, therefore, we applied ED-PCR as a first screening on vaccines for domestic fowl use, as well as on vaccines for other mammalian use for which the current mycoplasma contamination test has not been conducted at the national assay.

The procedure of ED-PCR was carried out as follows. A 500 μl sample of each liquefied or lyophilized vaccine, prepared according to directions, was used as a sample, and centrifuged at 15,000 × g at 4°C for 20 min. The pellet was suspended in 40 μl of lysis solution, which contained 50 mM KCl, 2.5 mM MgCl₂, 0.01% (w/v) gelatine, 0.45% (w/v) Noidet-P40, 0.45% (v/v) Tween 20, 0.2 mg/ml Proteinase K, and 10 mM Tris-HCl (pH 8.3). The mixture was incubated at 60°C for 20 min and 94°C for 10 min to inactivate Proteinase K by a DNA thermal cycler (Temp Control System PC-700, Asteck Co., Ltd., Japan). The PCR mixture solution (10 μl), which contained 50 mM KCl, 1.5 mM MgCl₂, 0.01% (w/v) gelatine, 1 mM dNTP mixture, 5 μg/μl each of primer, 0.75 units/μl of Tth DNA polymerase and 10 mM Tris-HCl (pH 8.9), was added to each tube. The oligonucleotide primers were primer 1 (5'-GCTGGCGTGAATACTGTTCT-3') and primer 2 (5'-CCCCACGTCTCCTGAGGGA-3') labeled with biotin N-hydroxysuccinimide ester and dinitrofluorobenzene, respectively. Amplification was achieved with 30 cycles of denaturation at 94°C for 30 sec, annealing at 50°C for 30 sec and extension at 72°C for 1 min, followed by a final extension at 72°C for 5 min. To identify the ED-PCR products an enzyme linked immunosorbent assay employed using streptavidin coated microtiter wells [7, 14]. After the enzymatic reaction, an OD₄₉₀ value of each well higher than 0.15 was recorded as positive for mycoplasma 16S rRNA genes.

For confirming specificity of ED-PCR, we applied a nested PCR to the samples which showed a positive reaction in ED-PCR. The procedure of the nested PCR was carried out as follows: 1 μl of ED-PCR product was added to the nested PCR solution which contained 78.5 μl of distilled water, 10 μl of 10 × PCR Mixture (Takara, Co., Ltd., Japan),
8 μl of 2.5 mM dNTP Mixtur (Takara), 0.5 μl of 5 unit of Taq DNA polymerase (Takara), and 1 μl of each nested PCR primer (20 pmol/μl). The sequence of nested PCR primers were as follows: primer 3 (5'-TCTCGGGTCTTGATACAGA-3') and primer 4 (5'-CCTGGTTACAGCTCACCC-3'). The program of nested PCR was carried out as follows: 30 cycles of one minute at 94°C, 40 seconds at 59°C, and 1 minute at 72°C, followed by seven minutes at 72°C. After amplification, 8 μl of each ET-PCR product was mixed with 2 μl of dye solution, then subjected to 3% (w/v) agarose gel electrophoresis and stained with 0.5 ng/μl ethidium bromide. A preliminary examination revealed that nested PCR can detect a single cell in vaccines contaminated with M. orale, M. hyopneumoniae, M. synoviae, and M. gallisepticum. The sizes of ED-PCR and nested PCR products were approximately 160 bp and 125 bp, respectively.

The direct culture method was also carried out [10]. Briefly, at least 2 bottles of vaccine were mixed, and 1 ml of the mix was inoculated into 100 ml of mycoplasma growth medium and incubated at 37°C for 14 days. On the third, seventh, tenth, and fourteenth day, the broth culture was inoculated onto agar plates. These agar plates were incubated at 37°C, under 5% (v/v) CO₂ condition. After 10 days incubation, we confirmed the specific mycoplasma colonies.

A total of 61 commercial monovalent and combined live vaccines were examined: 22 bovine vaccines, 25 porcine vaccines, 4 canine vaccines, a feline vaccine, a mink vaccine and 8 avian vaccines. The bovine vaccines included one of bovine ephemeral fever, three of IBR disease, five of infectious bovine rhinotracheitis (IBR), one of parainfluenza virus infection (PI), one of bovine respiratory syncytial virus infection, three of adenovirus infection, four of Akabane disease and four of IBR-bovine viral diarrhea-mucositis disease (BVD)-PI vaccines. The porcine vaccines included two kinds of hog cholera-swine erysipelas combined, six of hog cholera, four of transmissible gastroenteritis (TGE), two of porcine parvovirus infection (PPV), six of Japanese encephalitis, three of Aujeszky’s disease and two of Japanese encephalitis-PPV combined vaccines. The canine vaccines included distemper hepatitis combined, distemper-adenovirus type II infection-parainfluenza infection-parvovirus infection combined; distemper-adenovirus type II infection-parainfluenza infection-leptospirosis combined; and distemper-adenovirus type II infection-parainfluenza infection-parvovirus infection-leptospirosis combined vaccines. The feline vaccine was a feline viral rhinotracheitis-calicivirus-pneumovirina combined vaccine, the mink vaccine was a distemper vaccine, and the avian vaccines included Newcastle disease (ND), infectious bronchitis (IB), ND-IB combined, infectious laryngotracheitis, Marek’s disease, avian pox, avian encephalomyelitis and infectious bursal disease vaccines. These avian vaccines had passed the current test for the detection of mycoplasma contamination by the direct culture method at the national assay.

In the results with ED-PCR, the presence of mycoplasma DNA was apparent in some vaccines (Table 1). Of 61 vaccine samples examined, 22 (36.1%) showed a positive reaction implying Mycoplasma DNA contamination. Three of the 22 samples of bovine vaccines (13.6%), 17 of 25 porcine vaccines (68.0%), and 2 of 4 canine vaccines (50.0%) were positive. Moreover, the result of the nested PCR for all of these 22 vaccines also showed a positive reaction (Fig. 1). The vaccines which showed positive reactions for mycoplasma DNA were a bovine IBR-BVD-PI combined vaccine, an Akabane disease vaccine and an adenovirus infection vaccine; a porcine hog cholera vaccine, TGE vaccines, PPV infection vaccines, Japanese encephalitis vaccines, PPV Japanese encephalitis combined vaccines and Aujeszky’s disease vaccines; a canine distemper-hepatitis combined vaccine and a distemper-adenovirus type II infection-parainfluenza infection-parvovirus infection combined vaccine. The other veterinary vaccines were all negative for mycoplasma DNA in PCR.

In contrast, no viable mycoplasma was detected in any of 61 vaccine samples by the direct culture method (Table 1), and we could not detect any mycoplasma-like organisms in ED-PCR positive samples by electron microscopy (data not shown).

A positive reaction for PCR does not always indicate the existence of viable mycoplasma contamination in a vaccine manufacturing process. In general, PCR could react to both viable and inactivated microorganisms. The reason why viable mycoplasma could not be detected by the direct culture method in this study is not known. The possibility exists that the contaminated mycoplasma could not grow in the current medium, because some kinds of mycoplasma require a strictly specific nutrition [16]. Kobayashi et al. suggested that ED-PCR could detect some of the non-cultivable mycoplasma [7]. In addition, some antibiotics which are included in the vaccines as a preservative may inhibit the growth of contaminating mycoplasma. Therefore, the possibility that the contaminating mycoplasma may have already been inactivated or destroyed in the manufacturing process also exists. However, even though inactivated mycoplasma was present, we emphasize this is not a desirable situation in the quality control of the vaccines.

Table 1. Mycoplasma contamination test results for veterinary live virus vaccines

<table>
<thead>
<tr>
<th>Vaccine for animal</th>
<th>No. positive/No. tested</th>
<th>ED-PCR</th>
<th>Direct culture method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine</td>
<td>3/22 (13.6)</td>
<td>0/22 (0)</td>
<td></td>
</tr>
<tr>
<td>Porcine</td>
<td>17/25 (68.0)</td>
<td>0/25 (0)</td>
<td></td>
</tr>
<tr>
<td>Avian</td>
<td>0/8 (0)</td>
<td>0/8 (0)</td>
<td></td>
</tr>
<tr>
<td>Canine</td>
<td>2/4 (50.0)</td>
<td>0/4 (0)</td>
<td></td>
</tr>
<tr>
<td>Feline</td>
<td>0/1 (0)</td>
<td>0/1 (0)</td>
<td></td>
</tr>
<tr>
<td>Mink</td>
<td>0/1 (0)</td>
<td>0/1 (0)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>22/61 (36.1)</td>
<td>0/61 (0)</td>
<td></td>
</tr>
</tbody>
</table>
Unfortunately, the source of contamination is currently unknown because we used the final product of the vaccines as a sample. We suppose that such vaccines were contaminated with mycoplasma DNA but not viable mycoplasma. The importance of the existence of vaccines contaminated with viable pathogenic mycoplasma is apparent, especially in view of the chicken industry’s efforts to eradicate this organism from their flocks. In fact, the isolation of *M. gallisepticum*, a commercial infectious laryngotracheitis vaccine has been reported [3]. Furthermore, a serological test on an animal vaccinated with a mycoplasma contaminated vaccine may give false results [13].

In conclusion, mycoplasma DNA was detected at a high rate in veterinary live virus vaccines by PCR. We suggest that all veterinary live virus vaccines should be examined for the presence of mycoplasma or mycoplasma DNA at some stage of the vaccine manufacturing process, which will provide additional assurance of safety and quality control. In addition, PCR techniques may be useful for detection of mycoplasma contamination in veterinary live virus vaccines.

REFERENCES

9. Murakami, K., Minamide, W., Wada, K., Nakamura, E.,


