A survey of mycoplasma detection in veterinary vaccines

Denise H. Thornton

Nine live virus veterinary vaccines from six sources were found to be contaminated with mycoplasma. The vaccines were for use in canine, feline and avian species, and 53 batches of the products were at fault. The isolates were identified as* Mycoplasma hominis, M. arginini, M. orale, M. hyorhinis *and* M. gallinarum. *Investigation of the contamination rate of other batches or other products from the same source in some cases helped to determine the source of infection. Mycoplasma contaminants can be considered important not only because of their role as pathogens but also because they may indicate that insufficient care has been taken during vaccine manufacture or quality control.

Keywords: Contaminants; mycoplasma; veterinary vaccines

Introduction

Contamination of cell lines by mycoplasma is a well-documented phenomenon, large scale surveys revealing contamination rates of 5 to 50%[1–4]. The contamination may arise from the original tissue[3,5,6], the serum used in the culture medium[3,6], or from personnel handling the cultures[7]. Once established, cross contamination in the laboratory facilitates the widespread dissemination of the mycoplasma throughout other cell cultures[2,7]. Contamination by mycoplasma may not be detected on simple inspection even when present at high concentrations, as it may cause no cytopathic effect or turbidity.

Viral vaccines are generally produced in cell lines or occasionally in primary cell cultures or live animals. The use of antibiotics is permitted during vaccine manufacture because of the large scale of virus growth involved and the economic consequences that would ensue following casual bacterial contamination. However, the suppression of bacterial growth disguises and even encourages breakdown in aseptic technique, hence allowing opportunities for invasion by mycoplasma[8].

The risk of mycoplasma contamination can be reduced by using good vaccine manufacturing techniques such as spatial or temporal separation of products, with sterilization procedures between production runs, establishment of stocks of seed viruses and cell seeds which can be carefully prepared and tested and the use of tested or sterilized serum. Simple and sensitive tests are now available to detect contamination by mycoplasma. However these may not yet be in routine use by quality control laboratories, so there is potential for the presence of mycoplasma in live virus vaccines. Various workers have reported the presence of mycoplasma in vaccines[9–13]. A survey in the USA indicated a 3% contamination rate in veterinary vaccines[14], but the rate in a more recent survey was lower[15].

This paper presents an account of the discovery of mycoplasma in live virus veterinary vaccines licensed for use in the UK.

Materials and methods

Vaccines

Live virus vaccines were selected from batches of products licensed for use in the UK, samples of which are routinely submitted to this laboratory. The survey covered a 12 year period.

Detection of mycoplasma

The modified C medium used for isolation of mycoplasma from vaccines and the methods of isolation and identification have been described by Woods[16].

Results

In all the instances of mycoplasma contamination described below, the vaccines had been tested and declared satisfactory by the manufacturers of the products. Further collaborative work revealed defects in the method used for mycoplasma detection or more usually in the ability of the manufacturers' media to adequately support the growth of a range of mycoplasma species. In all cases, the batches in question were withdrawn from the market, and further supplies were suspended until the problems were resolved.

Company 1

M.hominis was detected in seven batches of a vaccine against Marek's disease of chickens. After confirmation by the manufacturers, they then discovered the vaccine seed virus to be contaminated. A new seed virus was

Ministry of Agriculture Fisheries and Food, Central Veterinary Laboratory, Weybridge, Surrey, UK.
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prepared, but this also gave rise to contaminated vaccine, so further efforts were needed to obtain an uncontaminated seed virus.

Company 2

M.arginini was found in 18 batches of canine distemper or distemper–hepatitis vaccine. Using the modified C medium, the manufacturers then found the distemper seed virus to be contaminated. Antibiotic treatment and filtration was successful in purifying the virus seed. A low level of contamination with *M.orale* was then detected in the new cell line used for virus growth, and antibiotic treatment was used to eliminate this.

Company 3

M.hominis was found in six batches of two other vaccines used to protect against canine distemper. After confirming the contamination using the modified C medium, and demonstrating it in the seed virus the manufacturer obtained new cells and seed virus which were mycoplasma free.

Company 4

M.gallinarum was found in two batches of live Newcastle disease vaccine. The manufacturer was unable to determine the source of contamination, but contamination was not found in subsequent batches.

Company 5

M.orale was found in 15 batches of canine distemper or distemper–hepatitis vaccine. The manufacturer found that the cell seeds were contaminated. New cells were obtained but they gave rise to three further batches of contaminated vaccine because the seed viruses had been prepared in contaminated cells. The seed viruses were then cloned in the presence of antibiotic and were then found to be satisfactory.

Company 6

M.hyorhinis was found in two batches of feline panleukopenia vaccine. This was confirmed by the manufacturer using the modified C medium. The medium used by the manufacturer failed to grow *M.hyorhinis* or *Acholeplasma laidlawii*[16]. A further batch of vaccine contained *M.orale*. The cell line was successfully freed of contamination and no mycoplasma was detected in further batches of vaccine.

Discussion

Approximately 120 live virus vaccines are licensed for use in the UK. Nine products from six manufacturers were found to be contaminated by mycoplasma representing a product contamination rate of 7.5%. Altogether, 54 batches were found to be at fault.

The possible origins of contamination can be grouped into primary sources, such as the use of tissue or serum from infected animals or dissemination from infected personnel or adjacent animal houses, and secondary sources such as contaminated cell seeds or virus seeds or laboratory cross-contamination. Investigation of the source of vaccine contamination can be aided by determining the species identity of the mycoplasma and by investigating whether the contamination is restricted to a single batch of product or is found in several batches or in several different products from the same manufacturer.

In the instance of Company 1, the vaccine against Marek's disease was grown in primary chick embryo fibroblasts from specific pathogen free hens, and all vaccine batches examined were found to contain *M. hominis*, which is associated with man. This situation implies either repeated contamination by the manufacturing personnel, or a contaminated seed virus, and the latter was established as the case.

With Company 2, *M.arginini* was found in distemper and distemper–hepatitis vaccines which, at that time, were grown in primary canine kidney cells. *M.arginini* is commonly found in bovine serum[6,17] and the fact that many vaccine batches were at fault suggested that the seed virus had been prepared in contaminated medium.

Similar instances of contamination of seed stocks of cells and virus with the human-associated *M.hominis* and *M.orale* were found with Companies 3 and 5.

With Company 4, the vaccine was produced in chicken embryos derived from specific pathogen free flocks and only two consecutive batches of vaccine were found to be at fault. Although production flocks are not required to be shown to be free of *M.gallinarum*, this organism is egg transmitted, and this may have been the cause of the problem. Alternatively, aerial transmission may have occurred.

M.orale and *M.hyorhinis* were found in the first three batches of product from Company 6 that were presented to this laboratory. *M.hyorhinis* is associated with pigs, and is potentially derived from trypsin used in cell cultures[18]. Its presence therein has never been established and it may in fact be derived from bovine serum following cross contamination in the slaughterhouse[19].

The significance of the presence of mycoplasma in vaccines in terms of danger to the recipient or the handler of the vaccine warrants discussion. Many *Mycoplasma* spp have definite pathogenic effects, but in some cases their role in causing or exacerbating disease remains to be established. However, it cannot be assumed that they are harmless. Some mycoplasma are known to be susceptible to infection with phages and it has been proposed that such infections may affect the pathogenicity of the mycoplasma; the pitfalls involved in trying to support this hypothesis are amusingly recounted by Clyde[20]. On the other hand, mycoplasma have been accused of playing the role of vectors of virus diseases[21]. The majority of *Mycoplasma* spp detected in this survey are probably not pathogenic for the species for which the vaccine is intended, and their role in human disease has not been established. It should be borne in mind that many vaccines are applied by aerosol or other routes such as intranasal, and are therefore disseminated into the environment where other species may be exposed. However in this survey, *M.gallinarum* was found in an avian vaccine. Although not pathogenic for chickens, the main target species, it may cause damage in other species in which such a vaccine might be used.

On a more practical level, mycoplasmas may affect the growth characteristics of the cell lines they infect[22] and they may alter virus yields[23] thus leading to poor control over quality and reduced productivity. It is possible that serological tests on an animal vaccinated with a mycoplasma contaminated vaccine may give

false results if a similarily contaminated antigen is used in the test[7,24]. They also represent a source of contamination to the rest of the vaccine plant.

The detection of mycoplasma contamination also provides an insight into manufacturers' production and quality control procedures. Probably in most of the instances recounted here, the mycoplasma may have been present in the substrates or virus seeds when they were obtained by the manufacturer. Such materials require careful screening before use, and it must be ensured that the culture media are able to support the growth of a small inoculum of a range of mycoplasma species which have not been adapted to growth in the laboratory. None of the companies involved had any difficulty in growing their contaminants once suitable media were used.

Moreover, because the presence of mycoplasma generally results from a breakdown in aseptic technique or use of unsatisfactory ingredients at some stage of vaccine development or production, it suggests the possibility of other forms of contamination which are associated with the same sources as the mycoplasma.

Bovine serum which has been produced or treated unsatisfactorily or tested inadequately may contain in addition to mycoplasma, viruses[25], fungi[26], bacteria[26,27], bacteriophages[28] and endotoxin[27]. Some of these agents may also be present in tissues used as a source of cell cultures[29]. All these types of contaminating agents have been detected in vaccines as a result of using such materials[30–32].

Laboratory cross-contamination can result not only in the spread of mycoplasma and other micro-organisms but also of live cells and this can result in overgrowth and replacement of a slower-growing cell line by one that may not be suited for vaccine production in terms of safety or ability to support virus growth[33]. Cell line cross contamination can be detected by various methods[8] and the technique of isoenzyme analysis can usefully be applied to cell lines.

It is therefore worthwhile screening mycoplasma-contaminated seed stocks for the presence of all these other contaminants if it is essential to continue their use.

Mycoplasma contamination of vaccines may best be prevented by careful screening of materials before introduction into the production area. Mycoplasma are sometimes found in unexpected reagents such as serum-free medium[34], or tritiated thymidine[35], so such materials should not be disregarded. Serum has been found to contain mycoplasma before and after filtration as high pressure filtration permits their passage[17]. It is recommended that large volumes of serum are used in cultural tests, but care must be taken in case inhibitory substances are present which would hinder their detection[36].

Materials of animal origin may be treated in a manner that will destroy mycoplasma, but undesirable antigens may remain. Trypsin can be subjected to treatment at pH 1[37], and serum can be irradiated, exposed to heat[17] or treated with inactivants such as formalin[38], phenol[38], betapropiolactone[38], binary ethyleneimine[39] or peracetic acid[40], which, under suitable conditions, will destroy mycoplasma.

Cells should ideally be cultured in the absence of antibiotics so that a breakdown in aseptic technique can be detected and investigated[2]. Once contaminated, various treatments of cell or virus seeds can be attempted.

Growth in the presence of specific antiserum was successful[41,42], as was growth in the presence of normal guinea-pig or rabbit serum[43]. Passage of a tumorigenic cell line in histocompatible mice for several weeks allowed the selection of a tumour consisting of mycoplasma-free cells[44]. *In vitro* culture in the presence of mouse macrophages was also successful[45]. However, these methods incur the risk of contamination with other agents from the animals or serum and so cannot be recommended. Antibiotic treatment is sometimes successful, but resistant strains may emerge[41].

Marcus[46] described a technique which involved selective killing of infected cells. The cultures were treated with a pyrimidine analogue which was incorporated by the mycoplasma, rendering the infected cells photosensitive. These cells were then destroyed by exposure to ultraviolet light, and mycoplasma-free cells could be cloned from the remainder. This technique has been used successfully by others[47,48] and it may be worthy of consideration by vaccine manufacturers particularly if the cell or virus seeds are irreplaceable. However, great care must be taken, in terms of repeated cloning, subculturing and testing, to ensure that the treatment has been successful.

Many methods have been developed for the detection of mycoplasma. The cultural method can be very sensitive but the growth initiation and support of the medium must always be very carefully controlled[49]. Other methods[50] include enzyme studies, nucleic acid analysis, electron microscopic observation, fluorescent antibody staining, measurement of uridine to uracil uptake ratio or staining with fluorescent DNA-binding compounds. These techniques can be useful supporting methods for detecting those mycoplasma which cannot readily be grown in culture media. The most widely used and one of the simplest of these techniques is the use of Hoechst 33258 fluorescent DNA stain[51], and it is recommended that this technique is routinely used as an adjunct to the *in vitro* culture method, particularly to detect those mycoplasma with the more fastidious growth requirements.

Finally, mycoplasma contamination is one problem that will continue on into the area of 'new-technology' products, where cell lines are used for preparing monoclonal antibodies or genetically-engineered pharmaceuticals and vaccines.

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