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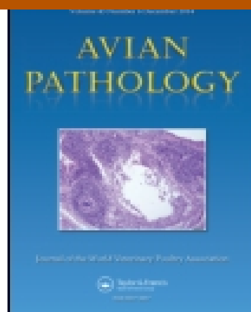

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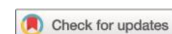

Influences of swab types and storage temperatures on isolation and molecular detection of *Mycoplasma gallisepticum* and *Mycoplasma synoviae*

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Abstract

Routine diagnosis of *Mycoplasma gallisepticum* (MG) and *Mycoplasma synoviae* (MS) is performed by collecting oropharyngeal swabs, followed by isolation and/or detection by molecular methods. The storage temperature, storage duration and the type of swabs could be critical factors for a successful isolation or molecular detection. The aim of this study was to compare the influence of different types of cotton tipped swabs stored at different temperatures, on detection of MG and MS. To achieve this, a combined use of traditional culture analysis (both agar and broth), with modern molecular detection methods was utilised. Performances of wooden and plastic shaft swabs, both without transport medium, were compared. Successful culture of *M. gallisepticum* was significantly more efficient from plastic swabs when compared to wooden, whereas no difference was seen for re-isolation of *M. synoviae*. Storage at 4 ^{o}C compared to room temperature also increased the efficiency of culture detection for both Mycoplasma species.

When stored at room temperature, PCR detection limits of both MG and MS were significantly lower for wooden compared to plastic swabs. The qPCR data showed similar detection limits for both swab types when stored at both temperatures. Results suggest that swabs with plastic shaft should be preferred for MG and MS detection by both culture and PCR. While a lower storage temperature (4°C) is optimal for culture recovery, it seems that both temperatures investigated here are adequate for molecular detection and it is the swab type which carries a greater influence.

Keywords: wooden swabs, plastic swabs, temperature, *Mycoplasma gallisepticum*, *Mycoplasma synoviae,* detection

Introduction

Mycoplasma gallisepticum (MG) and *Mycoplasma synoviae* (MS) are important poultry pathogens worldwide, both responsible for substantial economic losses. Oropharyngeal swabs collected from suspected infected flocks are routinely analyzed to confirm the presence of mycoplasmas by culture and/or molecular methodology. Sample storage temperature and the type of swab could influence successful detection (Christensen *et al.*, 1994; Zain and Bradbury, 1995; Zain and Bradbury, 1996; Daley et al., 2006; Ferguson-Noel *et al.*, 2012). The use of a suitable transport media (such as mycoplasma broth or charcoal) has been advised for transportation of samples.

As favorable transportation of samples for culturing may be the most important factor affecting successful detection of mycoplasmas (Drake *et al.*, 2005), it is important to consider field samples normally arrive at the laboratory 1-3 days after sampling. For PCR detection of MG or MS, results

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can be influenced by various factors, including the amount of DNA recovered, which could depend on type of swabs used, as well as the DNA extraction method (Brownlow *et al*, 2012).

The aim of this study is to compare two types of dry cotton swabs (wooden *versus* plastic shafts) which were stored at two different temperatures. Additionally, we investigated the effect of a longer duration between sample taking and laboratory processing. The influences of these factors on detection of MG and MS by isolation, and conventional and real-time PCR were assessed.

Materials and methods

Mycoplasma strains and culture

Two mycoplasma type strains were used throughout the study: MG PG31 and MS WVU 1853. Both strains were titrated using the viable counts method according to Miles *et al*. (1938) and expressed as colony-forming units (CFU)/ml. Briefly, strains were ten-fold diluted up to 10^{-7} in mycoplasma broth (MB). Then, 100 µl of each strain dilution were inoculated onto mycoplasma agar (MA) plates, using one plate per dilution. Both broth and agar media were prepared as previously reported (Bradbury, 1977; Zain and Bradbury, 1995). The plates were incubated at 37 °C in 5% CO_2 incubator for 7 days, before colonies were counted using a dissecting microscope. Titres were determined as 1.63×10^8 and 4.7×10^7 CFU/ml for MG and MS respectively.

Swabs

The performances of the following types of cotton tip dry swabs without transport medium were compared: wooden shaft and plastic shaft (Alpha Laboratories, Ltd, UK). For each mycoplasma species, each type of swab were used for culture or molecular analysis: swabs were stored at 4 °C and room temperature (RT; 21-23 °C), for 1, 2 and 3 days post inoculation (dpi). At each time

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point, 8 swabs were sampled of each type. In addition, cotton swabs with plastic shaft in Amies charcoal transport medium (Deltalab, Barcelona, Spain) were used for comparison.

Experimental design

MG and MS stock cultures with known titres were serially diluted (neat to 10^{-7}). Each series of wooden or plastic swabs, as well as the charcoal media swabs, were dipped into these broth dilutions for 15 seconds. Subsequently, swabs were stored at either 4 °C or RT as described above. Then, MG and MS recovery was attempted by culture and molecular methods (see below). Both culture recovery and molecular detection were repeated in triplicate for all samples.

Mycoplasma recovery by culture

Following storage at different temperatures, each of the dry (plastic and wooden) and charcoal swabs were plated onto MA and incubated at 37 °C in a 5% CO_2 incubator. After 7 days of incubation, colonies were quantified using a score from 0 to 4 as previously described (Ley *et al.*, 2003).

Molecular detection of mycoplasmas

Swabs intended for mycoplasma molecular detection were dipped into 600 µl of working solution D (4M guanidinium thiocyanate, 25mM sodium citrate, pH 7; 0.5% sarcosyl, 0.1M 2-mercaptoethanol) (Chomczynski and Sacchi, 2006) and stored at –20 °C for a minimum of three hours. DNA was then extracted using the DNA Mini kit (Qiagen, UK), according to manufacturer's instructions, and stored at –20 °C until use. The extracted DNAs were tested using a duplex PCR targeting the MG *mgc2* gene and the MS *vlhA* gene (Moscoso *et al.*, 2004). DNAs were also tested in duplicate using a commercial quantitative PCR (qPCR) kit for both MG and MS detection (BioChek, Netherlands) on the Rotor-gene Q platform (Qiagen, UK). Obtained Ct values were

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compared against a previously established standard curve (data not shown) of known concentrations, where relative log REU values were obtained.

Statistical analysis

Detection limits obtained from both culture or conventional PCR were analysed to identify statistically significant differences using Student *t*-test. A *P*-value <0.05 was considered statistically significant.

Results

Mycoplasma recovery by culture

M. gallisepticum: Culture of MG from swabs stored at RT showed that recovery was significantly more efficient for plastic (7.62×10^2 CFU/ml) than wooden (3.49×10^5 CFU/ml) swabs ($P<0.01$) (Figure 1A). Plastic swabs also had the greatest detection ability for MG culture from swabs stored at 4 °C (3.49×10^2 CFU/ml) compared to RT (7.62×10^2 CFU/ml) (Figure 1A), though there were no significant differences. The same was true at 2-3 dpi, as the plastic swabs showed greater detection ability compared to the wooden swabs, with only the high concentration sample (1.17×10^8 CFU/ml) showing a successful culture from wooden swabs by 3 dpi.

M. synoviae: By 1 dpi we were able to isolate MS to a minimum of 4.7×10^3 CFU/ml from plastic and wooden swabs stored at 4°C and plastic swabs stored at RT. The ability to re-detect MS from plastic swabs did not alter throughout for either temperature. No MS were isolated from wooden swabs stored at RT at 1 and 3 dpi, however high concentration samples were detected at 2 dpi (Figure 1B).

Molecular detection of mycoplasmas

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M. gallisepticum: At 1 dpi, minimum PCR detection limits were on average significantly lower for plastic ($3.49x10^3$ CFU/ml) compared to wooden ($7.62x10^4$ CFU/ml) swabs when stored at RT (*P*<0.05), whereas both swab types stored at 4 °C showed no difference in detection limits (Figure 1C). At later sampling points, the plastic swabs showed a greater ability to detect MG for both incubation temperatures. Similarly, the MG qPCR assay had greater detection capability when applied to plastic swabs stored at RT ($1.63x10^4$ CFU/ml) compared to wooden swabs ($1.63x10^5$ CFU/ml) at 1dpi. However, similar to PCR data, both swab types showed the same sensitivity at 4 °C (Figure 1E). At 2 and 3 dpi, only the wooden swabs stored at 4 °C were positive for MG, with all plastic swabs positive, although only at a higher concentration ($1.17x10^8$) compared to 1 dpi.

M. synoviae: By PCR, plastic swabs showed a lower minimum detection limit compared to wooden swabs when stored for 24 hours at 4 °C ($4.7x10^5$ CFU/ml and $1x10^6$ CFU/ml) and a significantly (*P*<0.05) lower result when stored at RT ($1x10^5$ CFU/ml and $2.2x10^6$ CFU/ml respectively) (Figure 1D). At 2-3 dpi, it was only possible to detect MG from the plastic swabs. In contrast, the MS qPCR showed the same detection sensitivity at 1 dpi for both types of swabs at RT ($1x10^4$ CFU/ml), but a greater efficiency when applied to plastic swabs at 4°C (plastic = $2.2x10^3$ CFU/ml; wooden = $4.7x10^3$ CFU/ml) (Figure 1F). Results at 2 and 3 dpi were similar to the PCR detection, with no wooden swabs positive for MS.

Discussion

Typically, when potentially infected poultry are sampled for mycoplasma detection, cotton tipped swabs are transported to the laboratory by the following day, however this may take several days depending on the location and method. While it is advised that transportation should also include ice or a cold pack to preserve sample integrity, it may not always be possible. For this reason, we investigated the influences of storage at two temperatures (4°C and room temperature), and several incubation times (1-3 dpi) on recovery of MG and MS using molecular and traditional

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culture methodologies. Previous work has highlighted the difference between swab types (Ferguson-Noel *et al.,* 2012; Zain and Bradbury, 1995); however we report the first study to combine the use of traditional culture analysis (both agar and broth), with modern molecular detection methods.

Findings from this study showed that dry plastic and charcoal swabs (both with a plastic shaft) had a similar ability to detect MG via culture when stored at 4 ^{o}C and RT. In contrast, while not significant, it appears that charcoal swabs were more effective for culturing MS when stored at 4 ^{o}C, with both plastic shaft swabs out-performing the wooden shaft. For both MG and MS, the dry plastic and charcoal swabs had a greater sensitivity to recover when stored at 4 ^{o}C, suggesting that transporting swab samples on ice is advantageous for successful detection (Zain and Bradbury, 1996).

In this study, the charcoal swabs showed a similar level of detection, irrespective of the storage temperature or duration, perhaps due to the preserving properties of charcoal medium, negating the effects of temperature fluctuations. The type of transport media and swab type used for sample preservation has shown to vary in ability to culture both aerobic and anaerobic bacteria (Tan *et al.,* 2014), with a possible reduction in recovery ability after 24 hours (Roelofsen *et al.,* 1999).

On culture of mycoplasmas, it appears that for both MG and MS, samples collected using wooden swabs and stored at RT could be detrimental for the detection of these organisms, either by isolation or PCR (especially for MS). In this study, although a reduced number of colonies were recovered for MG, no viable colonies were recovered for MS from wooden swabs stored at RT following either 1 or 3 dpi. Similarly, reduced levels of MG or MS detection were found in wooden

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swabs stored at RT when detection was attempted by PCR. The growth rate and viability of MG and MS can be also affected by the pH of the broth (Lin *et al*., 1983; Ferguson-Noel *et al.*, 2013) and it was previously hypothesized that greater humidity and lower temperature protected against the effect of low pH (Zain and Bradbury, 1996). This could be particularly true for MS, which may no longer be viable under a low pH (Ferguson-Noel *et al.*, 2013). In the present study, while the broth pH was not measured during incubation, a colour indicator alteration suggested an alteration in pH, alongside the difference in physical features of the wooden compared to the plastic swab (Ismail *et al.,* 2013).

Using molecular methods to detect MG, plastic swabs at RT initially displayed the greatest sensitivity. This could be related to permissive mycoplasma growth temperatures, which ranged from 20 to 45°C (Brown *et al.*, 2011). Previous work has reported that MG grown in mycoplasma broth and incubated at room temperature initially shows an increased titre up to 8 hours post inoculation, followed by a rapid decline in viability (Christensen *et al.*, 1994). Additionally, Zain and Bradbury (1996) demonstrated that the viability of MG on wet swabs reduces following 4 h of incubation at 24-26 °C. In the present study, molecular data showed that while the total genomic presence (viable and non-viable) increased, the number of viable colonies decreased when swabs were stored at RT. This was further emphasised at 2 and 3 dpi, as only the samples containing the highest concentrations of MG and MS were detected from plastic swabs, with no detections possible at RT (MG) or any temperature (MS) from wooden swabs.

In conclusion, results from the current study suggest that swabs with a plastic shaft should be preferred over the wooden shaft for MG and MS detection by culture, PCR and qPCR. While a lower storage temperature (4°C) is better for culture recovery, it seems that both temperatures

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investigated here are adequate for molecular detection, and the swab type is the bigger factor in determining a positive recovery.

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Conflict of Interest

All authors declare that they have no conflict of interest.

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List of figures

Figure 1. Comparison of each swab type following storage at 4 ^{o}C and room temperature (RT). (A) Culture efficiency for MG; (B) Culture efficiency for MS; (C) PCR detection of MG; (D) PCR detection of MS; (E) qPCR detection of MG; (F) qPCR detection of MS. Data shown as mean of the highest dilution producing a positive culture result, with standard error margins. Groups with the notation of 'a' indicate significant (*P*<0.05) differences within the same temperature, whereas 'x' indicates significant differences against the corresponding group at the different temperature.

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M. gallisepticum *M. synoviae*