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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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Published Version:

Influences of swab types and storage temperatures on isolation and molecular detection of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* / Ball C.; Felice V.; Ding Y.; Forrester A.; Catelli E.; Ganapathy K.. - In: AVIAN PATHOLOGY. - ISSN 0307-9457. - ELETTRONICO. - 49:1(2020), pp. 106-110. [10.1080/03079457.2019.1675865]

Availability:

This version is available at: <https://hdl.handle.net/11585/729061> since: 2020-02-19

Published:

DOI: <http://doi.org/10.1080/03079457.2019.1675865>

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Christopher Ball, Viviana Felice, Yichao Ding, Anne Forrester, Elena Catelli & Kannan Ganapathy (2020). Influences of swab types and storage temperatures on isolation and molecular detection of *Mycoplasma gallisepticum* and *Mycoplasma synoviae*, *Avian Pathology*, 49:1, 106-110.

The final published version is available online at:
<https://doi.org/10.1080/03079457.2019.1675865>

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3 Influences of swab types and storage temperatures on isolation and molecular detection of

4 *Mycoplasma gallisepticum* and *Mycoplasma synoviae*

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21

22 Abstract

23 Routine diagnosis of *Mycoplasma gallisepticum* (MG) and *Mycoplasma synoviae* (MS) is performed
24 by collecting oropharyngeal swabs, followed by isolation and/or detection by molecular methods.
25 The storage temperature, storage duration and the type of swabs could be critical factors for a
26 successful isolation or molecular detection. The aim of this study was to compare the influence of
27 different types of cotton tipped swabs stored at different temperatures, on detection of MG and
28 MS. To achieve this, a combined use of traditional culture analysis (both agar and broth), with
29 modern molecular detection methods was utilised. Performances of wooden and plastic shaft
30 swabs, both without transport medium, were compared. Successful culture of *M. gallisepticum*
31 was significantly more efficient from plastic swabs when compared to wooden, whereas no
32 difference was seen for re-isolation of *M. synoviae*. Storage at 4 °C compared to room
33 temperature also increased the efficiency of culture detection for both *Mycoplasma* species.
34 When stored at room temperature, PCR detection limits of both MG and MS were significantly
35 lower for wooden compared to plastic swabs. The qPCR data showed similar detection limits for
36 both swab types when stored at both temperatures. Results suggest that swabs with plastic shaft
37 should be preferred for MG and MS detection by both culture and PCR. While a lower storage
38 temperature (4°C) is optimal for culture recovery, it seems that both temperatures investigated
39 here are adequate for molecular detection and it is the swab type which carries a greater
40 influence.

41

42

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

45

46

47 Introduction

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

55

56 As favorable transportation of samples for culturing may be the most important factor affecting
57 successful detection of mycoplasmas (Drake *et al.*, 2005), it is important to consider field samples
58 normally arrive at the laboratory 1-3 days after sampling. For PCR detection of MG or MS, results
59 can be influenced by various factors, including the amount of DNA recovered, which could depend
60 on type of swabs used, as well as the DNA extraction method (Brownlow *et al.*, 2012).

61

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

66

67 Materials and methods

68

69 *Mycoplasma* strains and culture

70 Two mycoplasma type strains were used throughout the study: MG PG31 and MS WVU 1853. Both
71 strains were titrated using the viable counts method according to Miles *et al.* (1938) and

2 expressed as colony-forming units (CFU)/ml. Briefly, strains were ten-fold diluted up to 10⁻⁷ in
73 mycoplasma broth (MB). Then, 100 µl of each strain dilution were inoculated onto mycoplasma
74 agar (MA) plates, using one plate per dilution. Both broth and agar media were prepared as
75 previously reported (Bradbury, 1977; Zain and Bradbury, 1995). The plates were incubated at 37 °C
76 in 5% CO₂ incubator for 7 days, before colonies were counted using a dissecting microscope. Titres
77 were determined as 1.63x10⁸ and 4.7x10⁷ CFU/ml for MG and MS respectively.

78

79 **Swabs**

80 The performances of the following types of cotton tip dry swabs without transport medium were
81 compared: wooden shaft and plastic shaft (Alpha Laboratories, Ltd, UK). For each mycoplasma
82 species, each type of swab were used for culture or molecular analysis: swabs were stored at 4 °C
83 and room temperature (RT; 21-23 °C), for 1, 2 and 3 days post inoculation (dpi). At each time
84 point, 8 swabs were sampled of each type. In addition, cotton swabs with plastic shaft in Amies
85 charcoal transport medium (Deltalab, Barcelona, Spain) were used for comparison.

86

87 **Experimental design**

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

93

94 **Mycoplasma recovery by culture**

95 Following storage at different temperatures, each of the dry (plastic and wooden) and charcoal
96 swabs were plated onto MA and incubated at 37 °C in a 5% CO₂ incubator. After 7 days of

97 incubation, colonies were quantified using a score from 0 to 4 as previously described (Ley et al.,
98 2003).

99 Molecular detection of mycoplasmas

100 Swabs intended for mycoplasma molecular detection were dipped into 600 µl of working solution
101 D (4M guanidinium thiocyanate, 25mM sodium citrate, pH 7; 0.5% sarcosyl, 0.1M 2-
102 mercaptoethanol) (Chomczynski and Sacchi, 2006) and stored at -20 °C for a minimum of three
103 hours. DNA was then extracted using the DNA Mini kit (Qiagen, UK), according to manufacturer's
104 instructions, and stored at -20 °C until use. The extracted DNAs were tested using a duplex PCR
105 targeting the MG *mgc2* gene and the MS *vlhA* gene (Moscoso et al., 2004). DNAs were also tested
106 in duplicate using a commercial quantitative PCR (qPCR) kit for both MG and MS detection
107 (BioChek, Netherlands) on the Rotor-gene Q platform (Qiagen, UK). Obtained Ct values were
108 compared against a previously established standard curve (data not shown) of known
109 concentrations, where relative log REU values were obtained.

110

111 Statistical analysis

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

115

116 Results

117

118 Mycoplasma recovery by culture

119 *M. gallisepticum*: Culture of MG from swabs stored at RT showed that recovery was significantly
120 more efficient for plastic (7.62x10² CFU/ml) than wooden (3.49x10⁵ CFU/ml) swabs (P<0.01)
121 (Figure 1A). Plastic swabs also had the greatest detection ability for MG culture from swabs stored

122 at 4 °C (3.49×10^2 CFU/ml) compared to RT (7.62×10^2 CFU/ml) (Figure 1A), though there were no
123 significant differences. The same was true at 2-3 dpi, as the plastic swabs showed greater
124 detection ability compared to the wooden swabs, with only the high concentration sample
125 (1.17×10^8 CFU/ml) showing a successful culture from wooden swabs by 3 dpi.

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

131

132 **Molecular detection of mycoplasmas**

133 ***M. gallisepticum***: At 1 dpi, minimum PCR detection limits were on average significantly lower for
134 plastic (3.49×10^3 CFU/ml) compared to wooden (7.62×10^4 CFU/ml) swabs when stored at RT
135 ($P < 0.05$), whereas both swab types stored at 4 °C showed no difference in detection limits (Figure
136 1C). At later sampling points, the plastic swabs showed a greater ability to detect MG for both
137 incubation temperatures. Similarly, the MG qPCR assay had greater detection capability when
138 applied to plastic swabs stored at RT (1.63×10^4 CFU/ml) compared to wooden swabs (1.63×10^5
139 CFU/ml) at 1dpi. However, similar to PCR data, both swab types showed the same sensitivity at 4
140 °C (Figure 1E). At 2 and 3 dpi, only the wooden swabs stored at 4 °C were positive for MG, with all
141 plastic swabs positive, although only at a higher concentration (1.17×10^8) compared to 1 dpi.

142 ***M. synoviae***: By PCR, plastic swabs showed a lower minimum detection limit compared to wooden
143 swabs when stored for 24 hours at 4 °C (4.7×10^5 CFU/ml and 1×10^6 CFU/ml) and a significantly
144 ($P < 0.05$) lower result when stored at RT (1×10^5 CFU/ml and 2.2×10^6 CFU/ml respectively) (Figure
145 1D). At 2-3 dpi, it was only possible to detect MG from the plastic swabs. In contrast, the MS qPCR
146 showed the same detection sensitivity at 1 dpi for both types of swabs at RT (1×10^4 CFU/ml), but a

147 greater efficiency when applied to plastic swabs at 4°C (plastic = 2.2×10^3 CFU/ml; wooden =
148 4.7×10^3 CFU/ml) (Figure 1F). Results at 2 and 3 dpi were similar to the PCR detection, with no
149 wooden swabs positive for MS.

150

151 Discussion

152 Typically, when potentially infected poultry are sampled for mycoplasma detection, cotton tipped
153 swabs are transported to the laboratory by the following day, however this may take several days
154 depending on the location and method. While it is advised that transportation should also include
155 ice or a cold pack to preserve sample integrity, it may not always be possible. For this reason, we
156 investigated the influences of storage at two temperatures (4°C and room temperature), and
157 several incubation times (1-3 dpi) on recovery of MG and MS using molecular and traditional
158 culture methodologies. Previous work has highlighted the difference between swab types
159 (Ferguson-Noel et al., 2012; Zain and Bradbury, 1995); however we report the first study to
160 combine the use of traditional culture analysis (both agar and broth), with modern molecular
161 detection methods.

162

163 Findings from this study showed that dry plastic and charcoal swabs (both with a plastic shaft) had
164 a similar ability to detect MG via culture when stored at 4 °C and RT. In contrast, while not
165 significant, it appears that charcoal swabs were more effective for culturing MS when stored at 4
166 °C, with both plastic shaft swabs out-performing the wooden shaft. For both MG and MS, the dry
167 plastic and charcoal swabs had a greater sensitivity to recover when stored at 4 °C, suggesting that
168 transporting swab samples on ice is advantageous for successful detection (Zain and Bradbury,
169 1996).

170

171 In this study, the charcoal swabs showed a similar level of detection, irrespective of the storage
172 temperature or duration, perhaps due to the preserving properties of charcoal medium, negating
173 the effects of temperature fluctuations. The type of transport media and swab type used for
174 sample preservation has shown to vary in ability to culture both aerobic and anaerobic bacteria
175 (Tan *et al.*, 2014), with a possible reduction in recovery ability after 24 hours (Roelofsen *et al.*,
176 1999).

177

178 On culture of mycoplasmas, it appears that for both MG and MS, samples collected using wooden
179 swabs and stored at RT could be detrimental for the detection of these organisms, either by
180 isolation or PCR (especially for MS). In this study, although a reduced number of colonies were
181 recovered for MG, no viable colonies were recovered for MS from wooden swabs stored at RT
182 following either 1 or 3 dpi. Similarly, reduced levels of MG or MS detection were found in wooden
183 swabs stored at RT when detection was attempted by PCR. The growth rate and viability of MG
184 and MS can be also affected by the pH of the broth (Lin *et al.*, 1983; Ferguson-Noel *et al.*, 2013)
185 and it was previously hypothesized that greater humidity and lower temperature protected
186 against the effect of low pH (Zain and Bradbury, 1996). This could be particularly true for MS,
187 which may no longer be viable under a low pH (Ferguson-Noel *et al.*, 2013). In the present study,
188 while the broth pH was not measured during incubation, a colour indicator alteration suggested an
189 alteration in pH, alongside the difference in physical features of the wooden compared to the
190 plastic swab (Ismail *et al.*, 2013).

191

192 Using molecular methods to detect MG, plastic swabs at RT initially displayed the greatest
193 sensitivity. This could be related to permissive mycoplasma growth temperatures, which ranged
194 from 20 to 45°C (Brown *et al.*, 2011). Previous work has reported that MG grown in mycoplasma
195 broth and incubated at room temperature initially shows an increased titre up to 8 hours post

196 inoculation, followed by a rapid decline in viability (Christensen *et al.*, 1994). Additionally, Zain and
197 Bradbury (1996) demonstrated that the viability of MG on wet swabs reduces following 4 h of
198 incubation at 24-26 °C. In the present study, molecular data showed that while the total genomic
199 presence (viable and non-viable) increased, the number of viable colonies decreased when swabs
200 were stored at RT. This was further emphasised at 2 and 3 dpi, as only the samples containing the
201 highest concentrations of MG and MS were detected from plastic swabs, with no detections
202 possible at RT (MG) or any temperature (MS) from wooden swabs.

203

204 In conclusion, results from the current study suggest that swabs with a plastic shaft should be
205 preferred over the wooden shaft for MG and MS detection by culture, PCR and qPCR. While a
206 lower storage temperature (4°C) is better for culture recovery, it seems that both temperatures
207 investigated here are adequate for molecular detection, and the swab type is the bigger factor in
208 determining a positive recovery.

209

210 **Acknowledgement**

211 The authors would like to thank BioChek for the qPCR kits that were used for this study.

212

213 **Conflict of Interest**

214 All authors declare that they have no conflict of interest.

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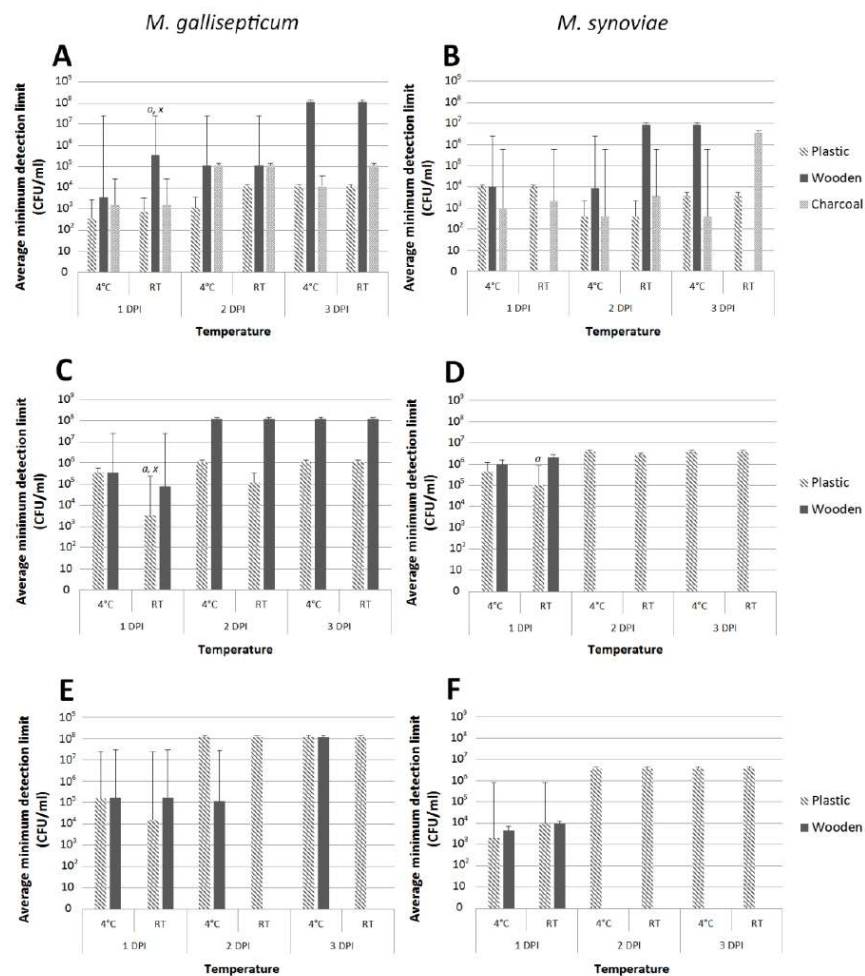
268 List of figures

269

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

276

277



Comparison of each swab type following storage at 4 °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.