Survival of Mycoplasmas Inoculated in Horse Sera

Hiroshi NAGATOMO, Yasuaki TOKITA, and Takamasa SHIMIZU
Division of Veterinary Science, Faculty of Agriculture, Miyazaki University, Gakuen-Kibanadai, Miyazaki 889–21, Japan
(Received 11 June 1996/Accepted 27 January 1997)

ABSTRACT. Although it is known that commercialized bovine serum is sometimes contaminated with mycoplasmas, it is not clear whether mycoplasmas can survive in horse serum. In this study, as a preliminary examination of the survival of mycoplasmas inoculated in horse sera, the survivability of 8 strains of 7 mycoplasmas was tested. The results obtained reveal that two strains of M. bovis and M. gallisepticum were found to survive in non-heated and inactivated sera for 94 to 330 days at 30 or 37°C. Three strains of M. bovirhinis, M. gateae and A. laidlawii lived for 7 to 330 days depending upon the temperature maintained or pH of the serum. Strains of M. bovigenitalium and U. diversum survived for a maximum of 8 days in all horse sera tested. Therefore, mycoplasmas are generally likely to survive for a long period in horse serum although the survival period depends on the species, strain and temperature. — KEY WORDS: equine serum, mycoplasma, survival.

It has been demonstrated that bovine serum is occasionally contaminated with mycoplasmas [3, 4, 6, 7], although information concerning the survival of mycoplasmas in horse serum (HS) is not available. HS is routinely supplemented to growth media for many mycoplasma species, though swine or bovine serum are used for some species. It has been proven that HS is most effective for enhancing the growth of the majority of mycoplasma species, without causing non-specific growth-inhibition [8]. When HS is taken from horse bloods collected at slaughterhouses where other animals are also bled, it is bled that the contamination of HS with mycoplasmas inhabiting to other animals or sewage occurs. Nevertheless, information on the survival of mycoplasmas contaminating, or inoculated into, HS is not yet available. Therefore, we tried to determined whether some mycoplasmas contaminating HS could grow or survive.

In this study, as a preliminary test, we examined the survival periods of animal mycoplasmas inoculated into horse sera. Although many mycoplasmas species or strains have a chance to be contaminants, we used 5 strains from bovine and sewage mycoplasmas as representative strains, and 3 strains from poultry and feline mycoplasmas as reference ones. They were Mycoplasma bovigenitalium PG11, M. bovis Donetta, M. bovirhinis PG43, Ureaplasma diversum strain B, Acholeplasma laidlawii PG8, M. gallisepticum PG31 and MR3, and M. gateae CS. For culturing of test organisms, Edward’s liquid medium containing 2.1% PPLO broth base (Difco Lab., Detroit, Mich., U.S.A.), 20% non-heated fresh HS (F-HS), 10% fresh yeast extract (Oriental Dry Yeast Co., Ltd., Tokyo, Japan) and 0.002% phenol-red was used. The solid medium used for colony counting was made from the liquid medium without phenol-red by supplementing 1% agar (Noble Agar, Difco Lab.). The pH of both media was adjusted to 7.5. Taylor-Robinson’s liquid medium [10] was used for culturing U. diversum strain. Commercially distributed HS (GIBCO Lab., New York, U.S.A.) was used as test media in this experiment.

The four HS prepared for the inoculations were: 1) F-HS (non-heated fresh horse serum), 2) Heated (H)-HS (heated at 56°C for 30 min), 3) F-HS adjusted to pH 6.0, and 4) H-HS adjusted to pH 6.0. Each organism was inoculated into three sets of test tubes (12 × 120 mm) which contained 2 ml of each HS and were capped with rubber stoppers.

Test organisms grown in liquid medium to the maximum growth stages were inoculated into 4 different HS preparations, to make a final concentration of 10^3 to 10^6 CFU (colony forming unit)/ml. The first set of tubes were kept at room temperature (RT), from April to March during which time the temperature ranged from 3 to 33°C during daytime, and the 2nd and 3rd sets of tubes were incubated at 30 and 37°C, respectively. These temperatures were decided upon by the growth and keeping temperatures for the organisms routinely used in the laboratory. Viable cells were counted on day 0, 7, 8, 28, 31, 35, 38, 53, 63, 94, 126, 154, 230, 295 and 330 post-inoculation. The counting of viable cells was carried out at intervals by inoculating a portion of 100 μl from each tube onto solid medium. The plates were then incubated at 37°C for 3 to 5 days in 5% CO2 incubator, and the number of colonies developed was counted under a microscope at 100-times magnification. The assay limit of this colony counting was 10^2 CFU/ml. Occasionally, tiny colony-like appearances were observed, however, since they did not grow after being transferred to liquid or solid medium, they were regarded as “artifacts”.

The results of viable cells recovered at intervals during the experimental period are shown in Fig. 1, and the final survival days of test strains are summarized in Table 1. As shown in Fig. 1(A), (B) and (C) and Table 1, the three strains of M. bovigenitalium, M. gateae and U. diversum perished in F-HS at 7 to 8 days at each of the three temperatures. The two strains of M. gallisepticum and M. bovis Donetta survived for 154 to 330 days, maintaining 10^3 to 10^4 CFU/ml of viable cells at 37°C. A. laidlawii did not survive longer than 8 days at 37°C, while it survived for 35 days at RT and 30°C. M. gallisepticum MR3 did not survive longer than 7 days at RT, while it survived for 126 and 330 days at 30 and 37°C with 10^3 to 10^6 CFU/ml of viable cells on the final day. M. bovirhinis PG43 existed for 35 days at 30°C while it did not live beyond 7 or 8 days at RT and 37°C.
The results obtained with H-HS are shown in Fig. 1(D), (E) and (F), and Table 1. It is clear that the results obtained are similar to those shown for F-HS, in which the strains of M. bovigenitalium, M. gateae and U. diversum survived for 8 days at most. Three strains of M. gallisepticum and M. bovis Donetta survived for 94 to 330 days at each temperature and maintained 10^2 to 10^5 CFU/ml of viable cells on the final day. A. laidlawii PG8 lived for 31 to 35 days at each temperature, although this strain did not survive more than 8 days when inoculated in F-HS at 37°C. M. bovirhinis PG43 lived for 35 days at 37°C, however this strain did not survive more than 7 or 8 days at RT or 37°C. The tendency was the same as the results shown for F-HS.

The results for F-HS adjusted to pH 6.0 are given in Fig. 1(G), (H) and (I), and Table 1. All strains except for M. bovis Donetta did not survive more than 7 days at RT. M. bovis Donetta survived for 154 days at RT and 295 days at 30°C, maintaining 10^3 CFU/ml of viable cells at each final day. In addition, M. bovis Donetta persisted for 38 days at 37°C, as shown in Table 1. M. gallisepticum PG31 tended to die out in a short period at RT and 30°C, whereas strain MR3 of the same species survived for 35 days at 30°C. None of strains of M. bovigenitalium, A. laidlawii and U. diversum survived beyond 8 days. The M. bovirhinis and M. gateae strains lived unexpectedly for 63 and 53 days at 37°C, respectively.
The results obtained for H-HS adjusted to pH 6.0 are shown in Fig. 1(J), (K) and (L), and Table 1. Out of the 8 strains tested, the three strains of *M. gallisepticum*, *M. bovis* and *M. gateae* survived for 53 to 154 days at RT, while the remaining 5 strains did not survive more than 7 days (Table 1). At 30°C, *M. gateae CS* survived for 126 days, and the two strains of *M. gallisepticum* lived for 35 to 94 days. *M. bovis* Donetta did not survive more than 8 days. *M. gateae* CS lived for 330 days at 37°C, maintaining 10^3 to 10^6 CFU/ml of viable cells, and survived for 94 to 330 days at each temperature. *M. bovis* Donetta existed for 230 days, although it’s viable cells on day 154 dropped below assay limit. The remaining 6 strains did not survive more than 8 days after inoculation.

Barile and Kern [4] isolated mycoplasmas, usually harbored in tissues of bovine and swine, from commercial bovine serum. Their results indicated that contamination may happened at the slaughterhouse or on the occasion of the filtration procedure. They also suggested that those mycoplasma strains could survive under maintained conditions such as at freezing point, at 4°C or higher temperatures. In the case of HS, we also considered that contamination with mycoplasmas is possible at the slaughterhouse, or on the occasion of filtering or using in the laboratory.

The present results indicate that *M. gallisepticum* and *M. bovis* inoculated into heated (H-HS) and non-heated HS (F-HS) are likely to survive for long periods (94 to 330 days) at RT, 30 and 37°C, although strain MR3 of *M. gallisepticum* perished after 7 days at RT. The strain MR3 may be sensitive to complement or the cidal-effect of fresh HS [9]. In addition, it was shown that *A. laidlawii* PG8 was found to exist for nearly a month (31 to 35 days) at all three temperatures, except in F-HS at 37°C. *M. bovirhinis* PG43 also existed for 35 days in both F-HS and H-HS at 30°C, although it died out at 8 days at RT and 37°C. The strains of *M. bovigenitalium, M. gateae* and *U. diversum* did not survive more than 8 days at all three temperatures. From these results, we concluded that some species of mycoplasmas could survive in H-HS or F-HS for long periods, especially pathogen mycoplasmas such as *M. bovis* and *M. gallisepticum*. We considered that those long-surviving strains might also exist in tissues for a long period, and have a chance of infecting the hosts.

In our basic experiment, the pH of the commercialized HS ranged from 7.4 to 7.5, therefore, we tried to test the survivability of mycoplasmas at a lower pH such as 6.0. Ureaplasmas can grow in liquid medium with pH 6.0, although such a pH might be an unusual level for many mycoplasma species. The result indicate that the viable cell counts of the mycoplasmas tended to decrease at the lower pH, however, the *M. gateae* CS strain survived for a long period in fresh HS at pH 6.0. This is an interesting fact, and this phenomenon may form a specific character of the strain or species.

Incidentally, Okazaki and Fujiwara [9] reported the mycoplasma-cidal action of HS on some mycoplasmas in HS with added complement incubated for 24 hr. In this experiment, there were strains which died out during the early period and were not detected again. This tendency may be due to the cidal effect of HS. There were also some strains which were detected again at the next inspection. These strains might survive, having viable cells under the assay limit (<10^2 CFU/ml), in the HS or on the surface of the inside of the test tubes. The recovered colonies again showed the same type of colony of the original, although the serological identification for each colony was not carried out.

It has been known that several species of mycoplasmas are harbored in horses [1, 2, 5], but records on mycoplasma contamination of HS are not available. Although bovine, sewage, poultry and feline mycoplasma strains were used in

---

**Table 1. Final survival days: viable cells detected by continuous recovery tests**

<table>
<thead>
<tr>
<th>Horse serum</th>
<th>Temp.a</th>
<th><em>M. bovigenitalium</em></th>
<th><em>M. bovis</em></th>
<th><em>M. bovirhinis</em></th>
<th><em>U. diversum</em></th>
<th><em>A. laidlawii</em></th>
<th><em>M. gateae</em></th>
<th><em>M. bovis</em></th>
<th><em>M. bovirhinis</em></th>
<th><em>U. diversum</em></th>
<th><em>A. laidlawii</em></th>
<th><em>M. gateae</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh</td>
<td>RT</td>
<td>7</td>
<td>ND</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>35</td>
<td>94</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>30°C</td>
<td>7 35</td>
<td>8</td>
<td>295</td>
<td>35</td>
<td>8</td>
<td>35</td>
<td>94</td>
<td>126</td>
<td>8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>37°C</td>
<td>7 35</td>
<td>8</td>
<td>154</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>295</td>
<td>330</td>
<td>8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heated</td>
<td>RT</td>
<td>7</td>
<td>154</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>35</td>
<td>126</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>30°C</td>
<td>7 35</td>
<td>8</td>
<td>295</td>
<td>35</td>
<td>8</td>
<td>35</td>
<td>94</td>
<td>295</td>
<td>8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>37°C</td>
<td>7 35</td>
<td>8</td>
<td>154</td>
<td>8</td>
<td>8</td>
<td>31</td>
<td>295</td>
<td>230</td>
<td>8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fresh</td>
<td>RT</td>
<td>7</td>
<td>154</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>35</td>
<td>8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH 6.0</td>
<td>7 35</td>
<td>8</td>
<td>295</td>
<td>35</td>
<td>8</td>
<td>8</td>
<td>35</td>
<td>ND</td>
<td>8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heated</td>
<td>RT</td>
<td>7</td>
<td>154</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>53</td>
<td>94</td>
<td>7</td>
<td>43</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>pH 6.0</td>
<td>7 53</td>
<td>8</td>
<td>295</td>
<td>35</td>
<td>8</td>
<td>8</td>
<td>94</td>
<td>35</td>
<td>126</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

this study, further experiments using mycoplasma strains which specifically inhabit horses should be encouraged. The results of the present study suggest that mycoplasmas contaminating or inoculated into HS can survive for long periods, though this depends on the species, strain and the temperature maintained.

REFERENCES