Microplate Sugar-Fermentation Assay Distinguishes *Streptococcus equi* from Other Streptococci of Lancefield’s Group C

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Lancefield’s group C streptococci often contaminate test samples taken from open lesions of the equine disease strangles, and it is difficult to distinguish the causal agent, *Streptococcus equi* (*S. equi*), from these other streptococci on the isolation plate. Here we have developed a microplate sugar-fermentation assay that distinguishes *S. equi* from other streptococci of Lancefield’s group C. Within 18 hr of incubation, the assay distinguished between 19 strains of *S. equi*, 171 strains of *Streptococcus zooepidemicus* and 19 strains of *Streptococcus equisimilis*, which were isolated from clinical horse samples and identified by API 20 STREP and Western immunoblotting. Moreover, this microplate assay can simultaneously test up to 24 samples, and is therefore valuable for the diagnosis of strangles.

**Key words:** Strangles, *Streptococcus equi*, sugar-fermentation assay

Strangles is a highly contagious equine disease that causes pyrexia, nasal discharge and secretion of pus from the lymph nodes [4]. Recently, the number of outbreaks of strangles increased in Hidaka district, which is the main racehorse-breeding area of Japan, and therefore better control of the disease has been demanded [1]. Strangles is caused by *Streptococcus equi* subsp. *equi* (*S. equi*), belongs to β-hemolytic and Lancefield’s group C streptococcus, and is diagnosed following the isolation and identification of *S. equi* from abscesses and mucopurulent nasal discharge.

β-Hemolytic and Lancefield’s group C streptococci include two other bacteria, *Streptococcus equi* subsp. *zooepidemicus* (*S. zooepidemicus*) and *Streptococcus dysgalactiae* subsp. *equisimilis* (*S. equisimilis*). These two bacteria are not only isolated from various clinical samples from horse, but are also detected together with *S. equi* in strangles lesions as a secondary opportunistic pathogen; in addition, it is difficult to distinguish *S. equi* from *S. zooepidemicus* and *S. equisimilis* by analyzing only their colony formation. Identification of these three bacteria is traditionally performed on the basis of their respective fermentation of lactose, sorbitol and trehalose. Lancefield’s group C streptococci that fail to ferment any of these sugars are usually identified as *S. equi*, those that ferment lactose and sorbitol are identified as *S. zooepidemicus*, and those ferment trehalose are identified as *S. equisimilis* [2]. However, an atypical *S. equi* strain that ferments sugar has been isolated from suspected cases of strangles [5].

The aim of this study was to clarify the sugar-fermentation characteristics of β-hemolytic Lancefield’s group C streptococci isolated recently from horses in Japan, and to establish a reliable and quick diagnostic method of strangles using microplate fermentation assays.

We used 209 strains of β-hemolytic streptococci isolated from various clinical horse samples for this study. The strains were identified using STREPT LA (Denka Seiken, Tokyo) for Lancefield serological grouping, and API 20 STREP (bioMerieux-Vitek Japan, Tokyo) for biochemical and immunological tests. Western immunoblot analysis of M-like proteins from streptococci was performed by the method of Galan and Timoney [3]. A microplate sugar-fermentation assay was carried out using 96-well flat-bottom tissue-culture plates (Nalgen Nunc International, USA) and 150 µl fermentation broth consisting of purple broth base (Difco, USA), 10% inactivated horse serum, and

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1% of either trehalose, sorbitol, ribose, glucose, or H$_2$O as a negative control. Fermentation broth was dispensed into each well and the microplates were stored at −20°C before use.

The microplate sugar fermentation assay was carried out as follows. Each strain was cultured purely in Todd Hewitt broth (THB) (Difco) supplemented with 0.2% yeast extract and 10% inactivated horse serum. A drop of this culture medium was added to each sugar reaction well and incubated aerobically at 37°C. The Lancefield serological grouping test typed all isolates of β-hemolytic streptococci from horses as group C. Subsequently, 19 strains were identified as *S. equi*, 169 strains as *S. zooepidemicus* and 19 strains as *S. equisimilis*; two strains were not identified by API 20 STREP. These 209 isolates of β-hemolytic streptococci were also analyzed for M-like protein of *S. equi* by Western immunoblotting. A strong reaction between native M-like proteins and the rabbit antiserum was observed in extracts from the 19 isolates identified as *S. equi* by API 20 STREP (Fig. 1A). By contrast, in extracts from the 169 isolates identified as *S. zooepidemicus* and the 2 isolates unidentified by API 20 STREP, a strong reaction was observed between the rabbit antiserum and proteins with different molecular masses to that of the M-like protein of *S. equi* (Fig. 1B). In extracts from the 19 isolates identified as *S. equisimilis*, no proteins reacted strongly to the rabbit antiserum in Western immunoblots (Fig. 1C). Thus, these biochemical and immunological tests demonstrated that of the 209 isolates, 19 were *S. equi*, 171 were *S. zooepidemicus* and 19 were *S. equisimilis*. For the sugar-fermentation microplate assay, results were accepted as accurate after confirmation of a positive reaction in the positive control well and a negative reaction in the negative control well. The assay showed that of the 19 strains of *S. equi*, none fermented lactose, trehalose, sorbitol or ribose (Table 1). Of the 171 strains of *S. zooepidemicus*, 134 fermented lactose, sorbitol and ribose, but not trehalose; 36 strains fermented lactose and sorbitol, but not trehalose or ribose; and 1 strain fermented lactose and ribose, but not trehalose or sorbitol. All of the 19 strains of *S. equisimilis* fermented trehalose and ribose, but not lactose or sorbitol. The results of the reaction could be read within 18 hours of incubation.

None of the *S. equi* strains identified in this study fermented lactose, trehalose, sorbitol or ribose. On the one hand, these fermentation characteristics are the same as those described previously for a typical strain of *S. equi* [4]. On the other hand, atypical isolates of *S.
that ferment lactose and trehalose have been reported by Grant et al. [5]. These researchers therefore recommended that ribose should be used to identify S. equi, because all the S. zooepidemicus isolates in their study fermented ribose. We found here, however, that 36 of the 171 identified strains of S. zooepidemicus did not ferment ribose. Although the reasons why the two studies gave different results are not clear, one explanation might be that neither the S. equi strains isolated by us nor the S. zooepidemicus strains described by Grant et al. involved atypical strains of either streptococcus. This would then suggest that there are a few number of atypical strains of Lancefield’s group C β-hemolytic streptococci that cannot be identified by only a sugar-fermentation test, and that immunological or genetical tests are needed to identify atypical strains.

In summary, these results suggest that the microplate sugar-fermentation assay is useful for distinguishing S. equi from S. zooepidemicus and S. equisimilis, both of which can cause opportunistic infectious disease with clinical symptoms that resemble those of strangles. In addition, these bacteria may be isolated from open strangles lesions as secondary invaders. In diagnosing for strangles, therefore, it is important to analyze many isolated colonies of suspected β-hemolytic streptococci grown on agar plates. As this microplate assay can be stored in the freezer and can test up to 24 samples at the same time, it should clearly contribute to the diagnosis and prevention of strangles.

References


