NOTE ***Bacteriology***

ARDRA and RAPD Analyses of Human and Animal Isolates of *Streptococcus gallolyticus*

Eiki SASAKI[1)], Ro OSAWA[1)]*, Yosuke NISHITANI[1)] and Robert A. WHILEY[2)]

[1)]Department of Bioscience, Graduate School of Science and Technology, Kobe University, Rokko-dai 1–1 Nada-ku, Kobe 657–8501, Japan and [2)]Department of Clinical and Diagnostic Oral Sciences, Barts and the London, Queen Mary's School of Medicine and Dentistry, Turner St., London E1 2AD, United Kingdom

(Received 5 March 2004/Accepted 7 July 2004)

ABSTRACT. A total of 23 *Streptococcus gallolyticus* strains, consisting of 12 strains from feces of healthy animals and 11 from clinical cases of human or cow mastitis milk, were examined genealogically. Four strains of *S. bovis* "biotype II/1" and 3 strains of *S. equinus*, the closely related organisms to *S. gallolyticus*, were also analyzed for outgroup comparison. Neither the amplified ribosomal DNA restriction analysis (ARDRA) nor the randomly amplified polymorphic DNA (RAPD) analysis that had been designed to recognize *S. gallolyticus* strains virulent in pigeons could differentiate clinical strains from the others of *S. gallolyticus*. No correspondence between the DNA profile in either analysis and the host animal species was detected.

KEY WORDS: ARDRA, RAPD, *Streptococcus gallolyticus.*

J. Vet. Med. Sci. 66(11): 1467–1470, 2004

Streptococcus gallolyticus, which includes strains formerly identified as "*S. bovis* biotye I" and "*S. bovis* biotype II/2", can be distinguished from other related taxa or biotypes (i.e. *S. equinus*, and *S. bovis* "biotype II/1"), based on not only the results of DNA-DNA reassociation experiments [12] but also by amplified ribosomal DNA restriction analysis (ARDRA) [15]. Recently, Schlegel *et al.* [16] have proposed reclassifying *S. gallolyticus* strains formerly designated as "*S. bovis* biotype II/1" and as "*S. bovis* biotype II/2", and "*S. macedonicus*" strains into 3 subspecies, *S. gallolyticus* subsp. *gallolyticus* subsp. nov., *S. gallolyticus* subsp. *pasteurianus* subsp. nov. and *S. gallolyticus* subsp. *macedonicus* subsp. nov, respectively. Most of the strains belonging to the former 2 subspecies can decarboxylate gallate as well as produce tannase, by which a hydrolyzable tannin (i.e. gallotannin) is hydrolyzed to release gallic acid, which is subsequently decarboxylated to pyrogallol [12].

Although *S. gallolyticus* subsp. is often found in the gut-microflora of various animals [13], it has been reported to cause mastitis in cattle [7], septicemia in pigeons [4], and meningitis, sepsis and endocarditis in humans [3, 10, 14]. Devriese *et al.* [5] demonstrated that most *S. gallolyticus* strains isolated from clinical cases were distinct from those isolated from the alimentary tracts of ruminants by SDS-PAGE analysis of whole-cell proteins. With near full-length 16S ribosomal DNA sequence analysis, Herrero *et al.* [8] claimed that "*S. bovis* biotype I" is associated with endocarditis and malignant and premalignant colon lesions.

Recently, several studies have been undertaken in order to identify clinical strains of *S. gallolyticus* with certain genotypes. Baele *et al.* [1] have reported that the RAPD analysis distinguished pigeon isolates of *S. gallolyticus* into levels of the virulence. We deal here with genotypic characterization of *S. gallolyticus* strains, by employing the ARDRA and RAPD [1, 15] analyses in order to evaluate a possible genotypic distinction between clinical and subclinical strains.

Twenty-three strains of *S. gallolyticus* including 4 strains of *S. bovis* "biotype II/1" and 3 strains of *S. equinus* were used in the present study. The origins of the strains and the results of their phenotypic identifications are summarized in Table 1. For the genealogical analyses, whole genomic DNA from each isolate was prepared by the method of Marmur [11].

The ARDRA analysis was performed by the method described by Schlegel *et al.* [15]. Briefly, PCR amplification targeting a partial sequence of the rrn genes including the 16S-23S intergenic spacer region was performed by means of a DNA thermal cycler (GeneAmp PCR System 2700; Applied Biosystems) withprimers Ad (5'-AGAGTTTGATCMTGGCTCAG-3') and O24/3 (5'-CGACATCGAGGTGCCAAA-3'). The PCR products were then digested with one of the restriction endonucleases, *Hha*I, *Mbo*II or *Sau*3A. The restricted fragments were analyzed electrophoretically with 1.5% agarose gel and the fragment pattern was visualized by means of a UV illumination.

The ARDRA profiles of the strains are shown in Fig. 1. Two major clusters were detected at a similarity level of 75% or less from the dendrogram generated. One of the clusters consisted of *S. gallolyticus* strains and another cluster consisted of *S. bovis* "biotype II/1" and *S. equinus* strains. The cluster group of *S. gallolyticus* strains was subdivided into 2 subgroups at approximately 75% similarity. These subgroups consisted of the strains of "*S. bovis* biotype I" and "*S. bovis* biotype II/2", respectively, assigned by the API system. Several 16S rDNA-based phylogenic analyses reported elsewhere [2, 8] have indicated that "*S. bovis* biotype II/2" is distinct from "*S. bovis* biotype I". The evidence

* CORRESPONDENCE TO: DR. OSAWA, R., Department of Bioscience, Graduate School of Science and Technology, Kobe University, 1–1 Rokko-dai, Nada-ku, Kobe City 657–8501, Japan.

Table 1. Streptococcal strains used

Spiecies	Strain code[a)]	Isolated from:	Phenotypic identity by API 20 Strep
S. gallolyticus subclinical strains			
S. gallolyticus	ACM 3611^T	Koala feces	"*S. bovis* biotype I"
S. gallolyticus	ACM 3612	Koala feces	"*S. bovis* biotype I"
S. gallolyticus	ACM 3609	Brushtail possum feces	"*S. bovis* biotype I"
S. gallolyticus	ACM 3593	Kangaroo feces	"*S. bovis* biotype I"
S. gallolyticus	ACM 3850	Ringtail possum feces	"*S. bovis* biotype I"
S. gallolyticus	ACM 3849	Cow feces	"*S. bovis* biotype I"
S. gallolyticus	ACM 3551 (=NCDO 2572)	Cow rumen	"*S. bovis* biotype I"
S. gallolyticus	ACM 3852	Dog feces	"*S. bovis* biotype I"
S. gallolyticus	ACM 3853	Dog feces	"*S. bovis* biotype I"
S. gallolyticus	ACM 3848	Horse feces	"*S. bovis* biotype I"
S. gallolyticus	ACM 3847	Pig feces	"*S. bovis* biotype I"
S. gallolyticus	ACM 3845	Guinea pig feces	"*S. bovis* biotype I"
S. gallolyticus clinical strains			
S. gallolyticus	ACM 3546 (=NCDO 2019)	Cow mastitis lesion	"*S. bovis* biotype I"
S. gallolyticus	ACM 3547 (=NCDO 2088)	Cow mastitis lesion	"*S. bovis* biotype I"
S. gallolyticus	ACM 3548 (=NCDO 2134)	Cow mastitis lesion	"*S. bovis* biotype I"
S. gallolyticus	ACM 3552 (=NCDO 2631)	Human clinical case	"*S. bovis* biotype I"
S. gallolyticus	ACM 3874 (=F-1867)	Human clinical case	"*S. bovis* biotype I"
S. gallolyticus	ACM 3873 (=Vitec 1616)	Human clinical case	"*S. bovis* biotype I"
S. gallolyticus	ACM 3902 (=CDC 002)	Human clinical case	"*S. bovis* biotype I"
S. gallolyticus	ACM 3903 (=MG Eath)	Human clinical case	"*S. bovis* biotype I"
S. gallolyticus	ACM 3870 (=CDC 1723–81)	Human clinical case	"*S. bovis* biotype II/2"
S. gallolyticus	ACM 3869 (=CDC 2266–81)	Human clinical case	"*S. bovis* biotype II/2"
S. gallolyticus	ACM 3871 (=CDC 008)	Human clinical case	"*S. bovis* biotype II/2"
Strains received as *S. bovis*			
S. bovis	ACM 3539 (=NCDO597^T)	Cow feces	*S. bovis* "biotype II/1"
S. bovis	ACM 3540 (=NCDO 598)	Cow feces	*S. bovis* "biotype II/1"
S. bovis	ACM 3542 (=NCDO 1251)	Sheep rumen	*S. bovis* "biotype II/1"
S. bovis	ACM 3543 (=NCDO 2128)	Cow feces	*S. bovis* "biotype II/1"
Strains received as *S. equinus*			
S. equinus	ACM 3541 (=NCDO 1037^T)	Horse feces	*S. salivarius*
S. equinus	ACM 3544 (=NCDO 2445)	Horse feces	*s. equinus*
S. equinus	ACM 3545 (=NCDO 2446)	Horse feces	unidentifiable

a) ACM, Australian Collection of Microorganisms, Department of Microbiology, University of Queensland, St. Lucia, Queensland, Australia; NCDO, National Collection of dairy Organisms, Reading, United Kindom; CDC strains and MG Eath, donated by A. L. Coykendall, Department of Oral Diagnosis, School of Dental Medicine, University of Connecticut Health Center, Farmington, Connecticut, U.S.A.; Vitec 1616 and F-1867, donated by R. G. Knight, Department of Veterans Affairs Medical Center 10701, East London, United Kingdom

obtained from our ARDRA analysis further substantiated this genotypic distinction since the analysis is designed to detect variations not only in 16S sequences but also in 23S and the 16S-23S intergenic spacer sequences. Within the cluster of *S. gallolyticus*, however, the analysis could not differentiate the strains of clinical origin from those of subclinical origin. The ARDRA patterns were also not related to difference in the host species.

The RAPD assay as a whole genome analysis was performed with an oligonucleotide primer, OPM6 (5'-CTGGGCAACT-3'), according to the method described by Baele *et al.* [1] with some modifications in the PCR conditions. Briefly, the reaction mixture was heated at 94°C for 5 min, 35°C for 5 min, and 72°C for 5 min prior to 30 cycles of PCR amplification. One PCR cycle consisted of denaturation at 94°C for 30 s, primer annealing at 35°C for 1 min, and extension at 72°C for 1 min. After the 30 cycles for the amplification, the temperature was maintained at 72°C for 5 min and then chilled to 4°C until the next examinations. The profiles of the amplified products detected by electrophoresis in 2% agarose gel were analyzed in the same manner as the ARDRA.

The RAPD profiles of the derived dendrogram are shown in Fig. 2. "*S. bovis* biotype II/2" strains, ACM 3869, ACM 3870 and ACM 3871 formed a cluster with approximately the 80% similarity level. Four *S. bovis* strains, ACM 3539, ACM 3540, ACM 3542 and ACM 3543 formed a separate cluster at approximately the 50% level. The greater part of *S. gallolyticus* strains of "*S. bovis* biotype I" showed somewhat close relatedness (level of similarity, approximately 40%), but strains of, ACM 3548 and ACM 3609 formed individual sublines which were defined by lower levels of similarities (less than 25%). The evidence suggests that *S. gallolyticus* includes strains of diverse genetic properties.

Baele *et al.* [1] have reported that virulent strains of *S. gallolyticus* from pigeons have a common RAPD pattern,

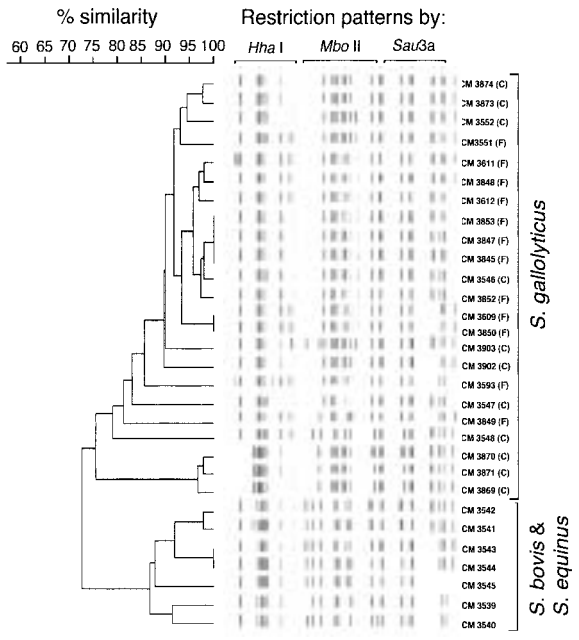

Fig. 1. ARDRA profiles of *S. gallolyticus*, *S. bovis* "biotype II/1", and *S. equinus* strains, and deduced dendrogram. The tandemly aligned ARDRA patterns of *Hha*I, *Mbo*II and *Sau*3A digests were converted to PICT files and entered into the GelCompar II program (Applied Maths) to generate a dendrogram based on the Dice coefficient [6], by the unweighted pair group method with 1% position tolerance. Parenthesized letters, C and F, indicate clinical and sub-clinical strains, respectively.

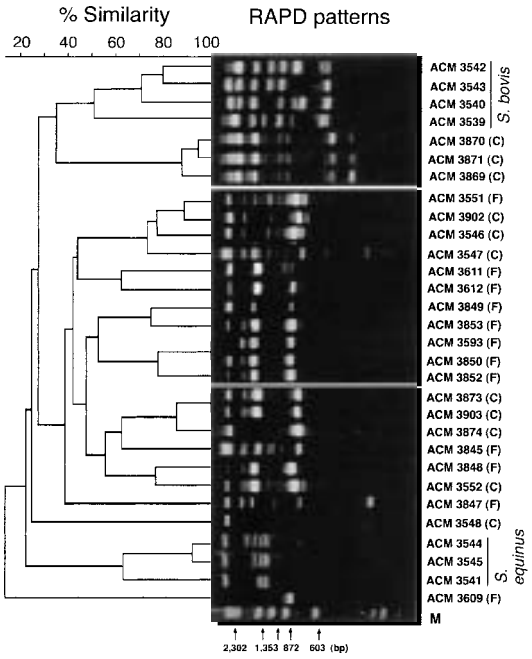

Fig. 2. RAPD profiles of *S. gallolyticus*, *S. bovis* "biotype II/1" and *S. equinus* strains, and deduced dendrogram. The dendrogram was generated in the same manner as described in Fig. 1. Parenthesized letters, C and F, indicate clinical and sub-clinical strains, respectively. Lane M, molecular size marker.

which was consisted of two intensified bands at 780 and 1,400 bp with several faint bands seldom found in avirulent strains of *S. gallolyticus.* Although the fragment patterns in the strains ACM 3849, ACM 3850 and ACM 3593 resembled those of the virulent pigeon strains, the majority of the RAPD profiles were noticeably heterogeneous. Thus there was no difference in the patterns between the clinical and subclinical strains. These facts suggest that the RAPD typing for distinguishing the virulence of *S. gallolyticus* strains cannot be generalized for the wider range of host animals. The RAPD profiles were also not related to difference in the host species.

In the present study, neither the ARDRA nor the RAPD analysis differentiated the *S. gallolyticus* strains of clinical origins from subclinical. This does not necessarily imply that all *S. gallolyticus* found in a normal gut-microflora is potentially pathogenic. As demonstrated elsewhere [9], clinical isolates have shown marked expression of an adhesion molecule sLe^x on the cell surface compared to fecal isolates. It is therefore tempting to speculate that limited numbers of genes regulating expression of such a molecule may be the key to identifying pathogenic *S. gallolyticus*. Further study is in progress to evaluate this possibility.

REFERENCES

1. Baele, M., Vanrobaeys, M., Vaneechoutte, M., De Herde, P., Devriese, L. A. and Haesebrouck, F. 2000. *Vet. Microbiol.* **71**: 103–111.
2. Clarridge III, J. E., Attorri, S.M., Zhang, Q. and Bartell, J. 2001. *J. Clin. Microbiol.* **39**: 1549–1552.
3. Cohen, L., Dunbar, S. and Clarridgem, J. E. 1997. *Clin. Infect. Disease.* **25**: 819–823.
4. Devriese, L. A., Uyttebroeck, E., Gevaert, D., Vandekerckhove, P. and Ceyssens, K. 1990. *Avian Aathol.* **19**: 429–434.
5. Devriese L. A., Vandamme, P., Pot, B., Vanrobaeys, M., Kersters, K. and Haesebrouck, F. 1998. *J. Clin. Microbiol.* **36**: 3520–3523.
6. Dice, L. R. 1945. *Ecology* **26**: 297–302.
7. Garvie, E. I. and Bramley, A. J. 1979. *J. Appl. Bacteriol.* **46**: 557–566.
8. Herrero, I. A., Rouse, M. S., Piper, K. E., Alyaseen, S. A., Steckelberg, J. M. and Patel, R. 2002. *J. Clin. Microbiol.* **40**: 3848–3850.
9. Hirota, K., Osawa, R., Kanitani, H., Nemoto, K., Ono, T. and Miyake, Y. 1996. *Lancet* **347**: 760.
10. Kupferwasser, I., Darius, H., Muller, A. M., Mohr-Kahaly, S., Westermeier, T., Oelert, H., Erbel, R. and Meyer, J. 1998. *Heart* **70**: 276–280.
11. Marmur, L. J. 1961. *J. Mol. Biol.* **3**: 208–218.
12. Osawa, R., Fujisawa, T. and Sly, L. I. 1995. *Syst. Appl. Microbiol.* **18**: 74–78.
13. Osawa, R. and Sly, L. I. 1992. *Syst. Appl. Microbiol.* **15**: 144–

147.
14. Ruoff, K., Millerm S. I., Garner, C. V., Ferraro, M. J. and Calderwood, S. B. 1989. *J. Clin. Microbiol.* **27**: 305–308.
15. Schlegel, L., Grimont, F., Grimont, P. A. D. and Bouvet, A. 2003. *J. Clin. Microbiol.* **41**: 657–666.
16. Schlegel, L., Grimont, F., Ageron, E., Grimont, P. A. D. and Bouvet, A. 2003. *Int. J. Syst. Evol. Microbiol.* **53**: 631–645.