



Association of Streptococcus pluranimalium with valvular endocarditis and septicaemia in adult broiler parents

Lise Hedegaard, Henrik Christensen, Mark Simon Chadfield, Jens Peter Christensen, Magne Bisgaard

► To cite this version:

Lise Hedegaard, Henrik Christensen, Mark Simon Chadfield, Jens Peter Christensen, Magne Bisgaard. Association of Streptococcus pluranimalium with valvular endocarditis and septicaemia in adult broiler parents. Avian Pathology, 2009, 38 (02), pp.155-160. <10.1080/03079450902737763>. <hal-00540144>

HAL Id: hal-00540144

<https://hal.science/hal-00540144v1>

Submitted on 26 Nov 2010

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



HAL Authorization



Association of *Streptococcus pluranimalium* with valvular endocarditis and septicaemia in adult broiler parents

Journal:	<i>Avian Pathology</i>
Manuscript ID:	CAVP-2008-0005.R3
Manuscript Type:	Original Research Paper
Date Submitted by the Author:	27-Dec-2008
Complete List of Authors:	Hedegaard, Lise; Copenhagen University, Veterinary Pathobiology Christensen, Henrik; Copenhagen University, Veterinary Pathobiology Chadfield, Mark; Copenhagen University, Veterinary Pathobiology Christensen, Jens; Copenhagen University, Veterinary Pathobiology Bisgaard, Magne; Copenhagen University, Veterinary Pathobiology
Keywords:	streptococcus, endocarditis, layer, PFGE

SCHOLARONE™
Manuscripts

Cavp-2008-0005.R3

Association of *Streptococcus pluranimalium* with valvular endocarditis and
septicaemia in adult broiler parents

Formatted: Font: (Default) Times New Roman, Not Italic, Complex Script Font: Times New Roman

Deleted: demonstrated as a new avian pathogen associated

L. Hedegaard, H. Christensen*, M. S. Chadfield, J. P. Christensen, M. Bisgaard

*Department of Veterinary Pathobiology, University of Copenhagen, Faculty of Life Sciences,
Stigbøjlen 4, DK-1870 Frederiksberg C, Denmark*

Running heading: *Streptococcus pluranimalium*

*To whom correspondence should be addressed. Tel: +45 35332783. Fax: +45 35332757.

E-mail: hech@life.ku.dk.

Received 15 January 2008

Abstract

The genus *Streptococcus* consists of more than 60 species, but only *S. equi* subspecies *zooepidemicus*, *S. gallolyticus* ssp. *gallolyticus*, *S. gallinaceus*, *S. dysgalactiae*, *S. mutans* and *S. suis* have been isolated from poultry. During investigations of the aetiology of increased mortality in broiler parent stock at the end of production, pure cultures of streptococcal-like organisms that could not be classified among these six species were obtained from 24 cases of septicaemia or valvular endocarditis and septicaemia. Phenotypic characterization using the API20 STREP kit identified the isolates as *Aerococcus viridans* (10), *Aerococcus urinae* (2), *Leuconostoc* species (4), *S. salivarius* (2), *S. bovis* II 3 (1), *Enterococcus avium* (3), *E. faecium* (1) or *Gemella morbillorum* (1). However, this identification was misleading as subsequent genetic investigations using pulse field gel electrophoresis and sequencing of 16S rRNA genes showed that 19 isolates were classified as *S. pluranimalium*, while the remaining isolates were *E. avium* (3), *E. faecium* (1) or *Lactobacillus* species (1). Misidentification by API20 STREP was related to the database provided by the manufacturer, as the phenotypic characteristics could identify these organisms as *S. pluranimalium*. The isolates of *S. pluranimalium* belonged to at least three different clones as determined by pulsed field gel electrophoresis of *Sma*I digested genomic DNA. The capacity that these isolates had to colonise the valvular endothelium was suggested by the occurrence of valvular endocarditis in 12 of 19 cases. Demonstration of the same clone in all four houses on a farm suggested the pathogenic potential of this organism.

Deleted: ,

Deleted: PFGE

Deleted: indicate

Deleted: s

Introduction

Application of new molecular techniques in bacteriology has resulted in numerous revisions of the genus *Streptococcus* over the last 25 years. In 1984, *Streptococcus faecium* and *S. faecalis* were transferred to the new genus *Enterococcus* (Schleifer & Kilpperbalz, 1984). A few years later the genus *Streptococcus* was divided into three genera, *Streptococcus*, *Lactococcus* and *Enterococcus* (Schleifer *et al.*, 1985). Since then many new species have been defined and today the genus *Streptococcus* contains more than 60 species (Euzéby, 1997; Kilian, 1998; Facklam, 2002; Hoshino *et al.*, 2005).

Deleted: 0

Deleted: ;

In animals the main habitats of streptococci are the skin and mucous membranes of the respiratory, gastrointestinal and urogenital tracts (Ruoff, 1992; Hirsh & Biberstein, 2004). Many species are commensals with pathogenic potential, while others are true pathogens in the sense that they spread from animal to animal and cause infection in non-immune individuals (Kilian, 1998). In poultry, *Streptococcus* species have been associated with local and systemic infections, including septicaemia and endocarditis (Wages, 2003). Species causing septicaemia include *S. dysgalactiae* (Messier *et al.*, 1993), *S. equi* subspecies *zooepidemicus* (Peckham, 1966), *S. gallinaceus* (Chadfield *et al.*, 2004), *S. gallolyticus* (Chadfield *et al.*, 2007), *S. mutans* (Ivanics *et al.*, 1984) and *S. suis* (Devriese *et al.*, 1994).

Deleted: ,

In addition, *Streptococcus* species have been associated with infections causing growth depression and increased mortality without obvious clinical signs (Chadfield *et al.*, 2004). According to Smyth & McNamee (2001), the *Streptococcus* species most commonly associated with disease in poultry is *S. equi* ssp. *zooepidemicus*. Reclassification of old species and recognition of many new species, combined with the lack of unambiguous characters for identification, has made classification and identification of streptococci challenging. In addition, many species seem to share several hosts. However, major genetic differences seem to exist between species, and a combination of phenotypic and molecular methods is likely to enable more certain diagnosis (Hoshino *et al.*, 2005).

Deleted: most common

During investigations of the aetiology of increased mortality in broiler parent stock at the end of production, pure cultures of streptococcal-like organisms that could not be classified among the six species regarded as pathogens of chickens were obtained from cases of septicaemia or valvular endocarditis and septicaemia. The aim of this study was to

characterize these organisms further to obtain an unambiguous diagnosis and possibly elucidate the epidemiology of disease caused by these organisms.

Materials and Methods

Flock Data. During 2004 and 2005, longitudinal studies were carried out on two consecutive broiler parent flocks on a farm in which endocarditis had been seen previously. The flocks contained approximately 42,000 hens and 3,400 roosters (Chadfield *et al.*, 2004). A total of 122 dead hens from the first flock, between 44 and 61 weeks old, were randomly selected from the four houses. In the subsequent flock a further 228 dead birds between 46 and 60 weeks of age were randomly selected from all four houses. In addition to this farm, reference strains were included from three other farms (Table 1). All birds were subjected to a comprehensive *post mortem* examination.

Deleted: previously affected by

Deleted:

Deleted: A

Deleted: randomly from the subsequent flock from all four houses

Bacteriology. Pure cultures of streptococcal-like organisms were obtained from cases with septicaemia, valvular endocarditis, and salpingitis, peritonitis, valvular endocarditis and septicaemia. Isolates were initially identified using standard methods (Wages, 1998). Isolates kept at -80°C were subsequently plated on blood agar base (CM0055, Oxoid) containing 5% sterile bovine blood (BA) and incubated overnight in sealed plastic bags at 37°C . Initial identification of pure cultures used assessment of colony morphology, haemolysis, Gram staining, catalase and oxidase tests, motility at 37°C , aerobic growth, catabolism of glucose in Hugh & Leifson medium and pyrrolidonyl-arylamidase activity (PYR) (Rosco, Denmark). Enzymatic reactions and fermentation of carbohydrates were determined using the API 20 STREP system according to the manufacturer's instructions (bioMérieux, La Balme les Grottes, France).

Pulsed Field Gel Electrophoresis (PFGE). Strains kept at -80°C were plated on BA and incubated overnight at 37°C . A single typical colony was sub-cultured overnight in 10 ml Brain Heart Infusion (BHI) broth (Oxoid) at 37°C with shaking. Some strains needed addition of 0.25% glucose and 10% chicken serum to the 10 ml BHI broth for growth. To measure and standardize cell density, the optical density (OD) of a solution containing 800

μ l SE buffer (75 mM NaCl, 25 mM ethylene-diamine-tetraacetic acid [EDTA], pH 7.4) and 200 μ l of culture in BHI broth was determined at 578 nm. Cells ~~therein a 3 ml sample of an~~ overnight culture in BHI broth were harvested by centrifugation for 10 min at 4°C. The supernatant was discarded and the pellet resuspended in 4.5 ml SE buffer. After further centrifugation, the supernatant was discarded and the pellet resuspended in sufficient SE buffer to obtain the same OD as was obtained previously. Five hundred microlitres of the suspension was mixed with 500 μ l agarose ~~solution~~ (0.02 g Megabase agarose [Bio-Rad], 1 ml SE-buffer) and dispensed into disposable plug molds (Bio-Rad). To release DNA from the cells the blocks were treated overnight at 37°C in 950 μ l lysozyme solution ~~at~~ pH 7.0 ~~containing~~ 2.5 mg hen egg white lysozyme (Roche), 1 ml phosphate buffer (0.1 M Na₂HPO₄, 2H₂O [Merck]) and 20% sucrose (J. T. Baker). After incubation, the blocks were washed for 30 minutes in 1 ml 10:10 TE buffer (10 mM Tris, 10 mM EDTA, pH 7.4) and incubated overnight at 56°C in 950 μ l proteinase K solution (0.5 mg proteinase K [Roche], 1 ml ES buffer (1% N-laurylsarcosine [Sigma-Aldrich], 0.5 M EDTA, pH 9.5). The blocks were then washed in cold TE buffer and incubated for 1 hour at room temperature. This was repeated 3 times. The blocks of purified chromosomal DNA were stored at 4°C in TE buffer until further use.

Deleted: of a three

Deleted: gel

Deleted: with

Deleted: and consisting

Deleted: of

PFGE was carried out as described by Chadfield *et al.* (2007) using *Sma*I for digestion of the DNA, an initial switch time of 1 sec, a final switch time of 15 sec, a potential difference of 5.6 V cm⁻¹ and an included angle of 120°C. The current was varied between 108 mA and 177 mA for the different gels. The gel was stained in a solution of ethidium bromide (Sigma) in deionised water (10 mg ethidium bromide ml⁻¹) for 15 minutes and afterwards de-stained in deionised water for 15 minutes, then photographed using ultraviolet light transillumination. A Low Range PFG Marker (N0350S, BioLabs, New England) was placed in ~~the~~ first, middle and last wells of the gel. The PFGE profiles produced from the clinical isolates were analysed using the unweighted pair-group method with arithmetic averages based on Dice similarity coefficients calculated using GelCompar II (Applied Maths).

Deleted: ,

Deleted: unweight

Deleted: th

Deleted: ed pair-group method, and comparisons made using

Sequencing of 16S rRNA genes. Sequencing of 16S rRNA genes of nine strains (20315H1HJ4, 20326H1L1, 20326H4L1, 20363H2HJ3, 20978H4L6, 21129H3HJ3, 21147H3L6, 21147H4L2 and 21192H3L2) representing the major clusters defined using

PFGE was done as described previously by Chadfield *et al.* (2007). The 16S rRNA gene sequences of two isolates from cases of septicaemia in layer hens (178 and C19672L28) and two isolates from epidemiologically unrelated cases in broiler parents (191 and 219) were included. Searches in GenBank (Benson *et al.*, 2006) were performed using BLAST (Altschul *et al.*, 1997). Pairwise comparisons for sequence similarity were performed using WATER within the EMBOSS package (Rice *et al.*, 2000). Multiple alignments were performed using CLUSTALX (Thompson *et al.*, 1997). The region corresponding to that between bases 71 and 1447 of the *E. coli rrnB* gene was examined.

Deleted: by

Maximum-likelihood analysis, including bootstrap analysis, was performed using fastDNAmI (Olsen *et al.*, 1994). The GenBank accession numbers for the sequences are shown in Figure 2. In addition, strains 21129H3HJ3, 21192H3L2 and 20326H4L1 were deposited under accession numbers EU391532, EU391533 and EU391534, respectively.

Deleted: 1

Results

Bacteriology. Streptococcal-like organisms were isolated from cases of septicaemia (11), valvular endocarditis and septicaemia (12), and salpingitis, peritonitis, valvular endocarditis and septicaemia (1). Only four isolates were obtained from the first flock, with the remaining isolates obtained from the second flock. In addition, four isolates from other farms were included (Table 1).

PFGE Analysis. Cluster analysis of PFGE profiles revealed two major clusters (A and B) with about 50% similarity (Figure 1). Twelve of 15 isolates formed one sub-cluster (A1) with 98% similarity, constituting a single clone according to Tenover *et al.* (1995). The remaining three isolates, identified as *E. avium* (A2) and *E. faecium* (A3), respectively, by phenotypic testing, formed a cluster with 73% similarity to each other. These three isolates had 66% similarity with subcluster A1, which was subsequently identified as *S. pluranimalium* by 16S rRNA sequencing. Cluster B included nine isolates within several subclusters (B1-4). Five of these isolates with 90% similarity constituted a second clone of *S. pluranimalium* (B1). This clone had 70% similarity to two other isolates (B2), both of which were subsequently classified as *S. pluranimalium* by 16S rRNA sequencing. Two

final isolates had distinct profiles (B3 and B4). The PFGE types of the isolates from flock 1 were different from those from flock 2 (Table 1, Figure 1).

Phenotypic Analysis. Twenty-four isolates tentatively classified as streptococcal-like organisms were characterized. Phenotypic characterization based upon the API 20 STREP system identified the isolates as *Aerococcus viridans* (10), *A. urinae* (2), *Leuconostoc* sp. (4), *S. salivarius* (2), *S. bovis* II 3 (1), *Enterococcus avium* (3), *E. faecium* (1) or *Gemella morbillorum* (1) (Table 1). This identification was not supported by PFGE or 16S rRNA sequence analyses. Phenotypic characteristics are shown in Table 1. Phenotypic characteristics, including those in the API20 STREP system that are useful for identification of *S. pluranimalium*, *E. avium* and *E. faecium* are shown in Table 2. Differences in pyrrolidonyl arylamidase and Voges-Proskauer reactions separate the streptococci and enterococci investigated. In addition, differences in hippurate hydrolysis, α -galactosidase and arginine dihydrolase production, and acid formation from D-mannitol, D-sorbitol, inulin, D-raffinose and starch separate *E. avium* from *E. faecium*. Isolates within PFGE subclusters A1, B1 and B2 could not be separated. All isolates identified as *S. pluranimalium* were strongly α -haemolytic on bovine blood agar plates, while isolates of *E. avium* and *E. faecium* produced weaker and greener α -haemolysis (Table 2).

16S rRNA Sequencing. A single isolate (20978H4L6) from the clonal PFGE subcluster A1 had 99.8% 16S rRNA gene sequence similarity with *S. pluranimalium* strain LMG14257, Acc. No. Y18026). However, two other isolates, 21147H3L6 and 20326H1L1, from PFGE subcluster B1 also had 99.8% similarity with strain LMG14257. Isolate 20315H1HJ4 from subcluster B2 had 99.0% 16S rRNA gene sequence similarity with *S. pluranimalium* and 16S rRNA gene sequence identity with an isolate (C19672L28) from a septicemic layer hen (Table 1, Figure 2). Isolates 20978H4L6 (A1) and 20315H1HJ4 (B2) had 99.9% 16S rRNA gene sequence similarity. Isolate 20363H2HJ3 (B2) had 98.9% sequence similarity with *S. pluranimalium* and 99.9% similarity with 21147H4L2 (B1), and was identical to an epidemiologically unrelated isolate (178) from a layer (Table 1). Isolate 20326H4L1 from PFGE subcluster B4, had 16S rRNA gene sequence similarities of 99.6 and 99.5% with the type strains of *Lactobacillus johnsonii* and *L. gasseri*, respectively (Acc. No. AJ002515 and AF519171). Two isolates, 21192H3L2 (A2) and 21129H3HJ3 (B3), which were classified as

Deleted: and

Deleted: Strains

Deleted: I

Deleted: I

Deleted: of

Formatted: Font: Italic

Deleted: identity

Deleted: with

Deleted: an

Deleted: isolate

Deleted: an epidemiologically unrelated isolate from

Deleted: Strain

Deleted: t

Deleted: y,

Deleted: between

E. avium by phenotyping, had 100% and 99.9% 16S rRNA gene sequence similarity, respectively, with the type strain (ATCC14025^T) of *E. avium* (Acc. No. DQ411811).

The close relationships between the ten isolates of *S. pluranimalium* compared by 16S rRNA gene sequencing were reflected in the inferred phylogeny (Figure 2). The monophyletic group including all these isolates and the reference strain of *S. pluranimalium* was supported by a high bootstrap value of 99%. *S. pluranimalium* was found to be closely related to the species *S. thoraltensis*, *S. gallinaceus*, *S. suis* and *S. gallolyticus*.

There was no correlation between the lesions and the PFGE subclones and there was no obvious difference between the lesions caused by *S. pluranimalium*, *E. faecium* or *E. avium*.

Discussion

S. pluranimalium was first described by Devriese *et al.* (1999). Based on sequencing of the 16S rRNA gene it is closely related to *S. thoraltensis* from pigs, *S. gallinaceus* from poultry and *S. suis* from pigs and birds (Figure 2). Whole cell protein analysis has confirmed that *S. pluranimalium* differs from closely related species of streptococci (Devriese *et al.*, 1999),

but phenotypic identification is complicated by variations in biochemical reactions.

Phenotypically, *S. pluranimalium* resembles *S. thoraltensis*, *S. hyovaginalis*, *S. suis* and *S. acidominimus*. In addition, *S. pluranimalium* may be confused with organisms in the *S. bovis* group. Colonies grown on blood agar plates are surrounded by a broad greenish zone of α -haemolysis, which makes distinction from *E. avium* and *E. faecium* difficult. *S. pluranimalium* has been isolated from several animal species and from different sites, including the genital tract (cervix and vaginal mucus), the mammary glands of cows and from the tonsils of cattle, a cat and goats. In avian species, it has been isolated from the lungs and crop of canaries (Devriese *et al.*, 1999). Of these species, only *S. suis* (Devriese *et al.*, 1994) and *E. avium* (Devriese *et al.*, 1991) have been isolated from birds previously.

In the study reported here *S. pluranimalium* isolated from adult broiler parents had growth characteristics and colonial morphology similar to those reported by Devriese *et al.* (1999), while some variations were seen in biochemical activity, including hydrolysis of

Deleted: d

Deleted: Phenotypically, it resembles *S. acidominimus* and

Deleted: b

Deleted: and

Deleted: however

Deleted: ,

Deleted: strains

Deleted: of

Deleted: y

hippurate, production of pyrrolidonyl arylamidase, β -glucuronidase, alkaline phosphatase and arginine dihydrolase. All the strains characterized by Devriese *et al.* (1999) hydrolysed hippurate but only two of our eighteen isolates hydrolysed hippurate. However, the bovine isolates varied in a number of phenotypic characteristics, highlighting the difficulties associated with phenotypic identification of this species (Devriese *et al.*, 1999).

Deleted: of our,

Recent evaluations of phenotypic and molecular methods for identification of non-haemolytic streptococci have indicated that identifications by commercial kits are correct in fewer than 50% of cases, but that this varies significantly between species (Hoshino *et al.*, 2005). In our study, correct identification of *S. pluranimalium* based upon classical phenotypic methods, including the API 20 STREP system, was not possible. Phenotypic characterization identified the 12 isolates from a single clone (A1) as *Aerococcus viridans*, *Leuconostoc* sp., *S. salivarius* and *S. bovis* II. Other isolates from clusters B1 and B2 were identified as *A. viridans*, *A. urinae*, *Leuconostoc* sp. and *S. salivarius* (Table 1).

Deleted: in

Phenotypically, both *E. avium* and *E. faecium* were weakly α -haemolytic, making them difficult to distinguish from *S. pluranimalium*. However, *E. avium* and *E. faecium* could be distinguished from *S. pluranimalium* by other characteristics listed in Table 1.

Deleted: separate

Deleted: separated

Analysis of PFGE patterns showed that *E. avium* and *E. faecium* were distinct from *S. pluranimalium*, with a similarity coefficient of less than 66%. PFGE demonstrated the existence of two clones, in addition to two other distinct isolates of *S. pluranimalium*, with an overall similarity of 60%.

Birds received for *post mortem* examination were between 44 and 61 weeks and age might be one of the predisposing factors for valvular endocarditis, since 12 of 19 birds infected with *S. pluranimalium* suffered from this condition. The bedding quality for the second flock was poor and distinctly different from that for the first flock. Poor bedding quality is a factor that may contribute to the prevalence of some bacterial diseases in poultry, but is difficult to quantitate.

Deleted: to

Deleted: out

Deleted: affected

Deleted: by

Deleted: in

Deleted: of

Deleted: quality

Deleted: well know

Deleted: contributing

Deleted: bacterial disease

Deleted: however, this parameter

Deleted: measure

Deleted: P

Deleted: therefore

Isolates of *S. pluranimalium* were obtained from both flocks and from all four houses. Given the high level of biosecurity at parent stock level, introduction of three different clones at the same time, or successively, would only be likely during placement of day-old birds at the rearing farm. Therefore preventive measures should address egg and hatchery hygiene. A number of virulence factors have been identified in streptococci associated with septicaemia and endocarditis (Kilian, 1998). Further investigations on the

existence, and importance, of virulence factors in *S. pluranimalium* are indicated. In conclusion, our study has demonstrated that *S. pluranimalium* is a novel avian pathogen associated with septicaemia or endocarditis and septicaemia in adult broiler parents.

Deleted: ,

Deleted: s

Formatted: English U.S.

References

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research*, 25, 3389-3402.

Benson, D. A., Karsch-Mizrachi, I., Lipman, D. J., Ostell, J. & Wheeler, D. L. (2006). Genbank. *Nucleic Acids Research*, 34, D16-20.

Formatted: Font: (Default) Times New Roman, 12 pt, Complex Script
Font: Times New Roman, 12 pt, English U.K.

Field Code Changed

Formatted: English U.K.

Chadfield, M. S., Christensen, J. P., Christensen, H. & Bisgaard, M. (2004). Characterization of streptococci and enterococci associated with septicaemia in broiler parents with a high prevalence of endocarditis. *Avian Pathology*, 33, 610-617.

Chadfield, M. S., Christensen, J. P., Decostere, A., Christensen, H. & Bisgaard, M. (2007). Geno- and phenotypic diversity of avian isolates of *Streptococcus gallolyticus* subsp. *gallolyticus* (*Streptococcus bovis*) and associated diagnostic problems. *Journal of Clinical Microbiology*, 45, 822-827.

Devriese, L. A., Hommez, J., Wjffels, R. & Haesebrouck, F. (1991). Composition of the enterococcal and streptococcal intestinal flora of poultry. *Journal of Applied Bacteriology*, 71, 46-50.

Devriese, L. A., Haesebrouck, F., Deherdt, P., Dom, P., Ducatelle, R., Desmidt, M., Messier, S. & Higgins, R. (1994). *Streptococcus suis* infections in birds. *Avian Pathology*, 23, 721-724.

Devriese, L. A., Vandamme, P., Collins, M. D., Alvarez, N., Pot, B., Hommez, J., Butaye, P. & Haesebrouck, F. (1999). *Streptococcus pluranimalium* sp. nov., from cattle and other animals. *International Journal of Systematic Bacteriology*, 49, 1221-1226.

Formatted: English U.K.

Euzéby, J. P. (1997). List of bacterial names with standing in nomenclature: a folder available on the Internet (URL: <http://www.bacterio.cict.fr/>). *International Journal of Systematic Bacteriology*, 47, 590-592.

Facklam, R. (2002). What happened to the streptococci: overview of taxonomic and nomenclature changes. *Clinical Microbiology Reviews*, 15, 613-630.

Hirsh, D. C. & Biberstein, E. L. (2004). *Streptococcus* and *Enterococcus*. In D. C. Hirsh, N. J. MacLachlan & R. L. Walker. *Veterinary Microbiology* 2nd edn (pp. 159-167). Ames: Blackwell Publishing.

Hoshino, T., Fujiwara, T. & Kilian, M. (2005). Use of phylogenetic and phenotypic analyses to identify nonhemolytic streptococci isolated from bacteremic patients. *Journal of Clinical Microbiology*, 43, 6073-6085.

Ivanics, E., Bitay, Z. & Glavits, R. (1984). *Streptococcus mutans* infection in geese. *Magyar Allatorvosok Lapja*, 39, 92-95.

Kilian, M. (1998). *Streptococcus* and *Lactococcus*. In A. Balows & B. I. Duerden (1998). *Topley and Wilson's Microbiology and Microbial Infections*, 9th edn (pp. 634-667). New York: Arnold.

Formatted: English U.S.

Messier, S., Quessy, S., Robinson, Y., Devriese, L. A., Homme, J. & Fairbrother, J. M. (1993). Focal dermatitis and cellulitis in broiler chickens: bacteriological and pathological findings. *Avian Diseases*, 37, 839-844.

Formatted: Dutch Netherlands

Olsen, G.J. Matsuda, H., Hagstrom, R. & Overbeek, R. (1994). Fast DNAm1: a tool for construction of phylogenetic trees of DNA sequences using maximum likelihood. *Computer Applications in the Biosciences*, 10, 41-48.

Peckham, M. C. (1966). An outbreak of streptococcosis (apoplectiform septicemia) in White Rock chickens. *Avian Diseases*, 10, 413-421.

Rice, P., Longden, I. & Bleasby, A. (2000). EMBOSS: The European Molecular Biology Open Software Suite. *Trends in Genetics*, 16, 276-277.

Ruoff, K. L. (1992). The genus *Streptococcus*. In A. Balows, H. G. Trüper, M. Dworkin, W. Harder & K.-H. Schleifer. *The Prokaryotes* 2nd edn (pp. 1450-1464). New York: Springer.

Schleifer, K. H. & Kilpperbalz, R. (1984). Transfer of *Streptococcus faecalis* and *Streptococcus faecium* to the genus *Enterococcus* nom. rev. as *Enterococcus faecalis* comb. nov. and *Enterococcus faecium* comb. nov. *International Journal of Systematic Bacteriology*, 34, 31-34.

Schleifer, K. H., Kraus, J., Dvorak, C., Kilpper-Bälz, R., Colling, M. D. and Fischer, W. (1985). Transfer of *Streptococcus lactis* and related streptococci to the genus *Lactococcus* gen. nov. *Systematic and Applied Microbiology*, 6, 183-195.

- Smyth, J. A. & McNamee, P. T. (2001). Staphylococci, streptococci and enterococci. In F. Jordan, M. Pattison, D. Alexander & T. Faragher. *Poultry Diseases* 5th edn. (pp. 163-169). London: W.B. Saunders.
- Tenover, F. C., Arbeit, R. D., Goering, R. V., Mickelsen, P. A., Murray, B. E., Persing, D. H. & Swaminathan, B. (1995). Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel-electrophoresis - criteria for bacterial strain typing. *Journal of Clinical Microbiology*, 33, 2233-2239.
- Thompson, J., Gibson, T.J., Plewniak, F., Jeanmougin, F. & Higgins, D.G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research*, 25, 4876-4882.
- Wages, D. P. (1998). Streptococcosis. In D. E. Swayne, J. R. Glisson, M. W. Jackwood, J. E. Pearson & W. M. Reed. *A Laboratory Manual for the Isolation and Identification of Avian Pathogens* 4th edn (pp. 58-60). Kennett Square, Pennsylvania: The American Association of Avian Pathologists.
- Wages, D. P. (2003). Streptococcosis. In Y. M. Saif, H. J. Barnes, J. R. Glisson, A. M. Fadly, L. R. McDougald & D. E. Swayne (2003). *Diseases of Poultry* 11th edn. (pp. 805-812). Ames: Iowa State Press.

Figure Legends

Figure 1. Dendrogram based on the unweighted pair-group method with arithmetic averages and Dice similarity coefficients between *Sma*I PFGE profiles. The percentage similarity in regard to clusters formed is shown at the top scale and for specific clusters at each node on the dendrogram.

Figure 2. Phylogenetic relationships between isolates of *S. pluranimalium* from broiler parents and related species of *Streptococcus* based on maximum likelihood analysis of 16S rRNA gene sequences. The percentage support for monophyletic groups by bootstrap-analysis are indicated at specific branches. The strains in bold were sequenced in our investigation and strains in parentheses have identical sequences. The scale bar represents branch length based on the maximum likelihood analysis.

Deleted: legend to the figures.

Formatted: Font: Bold

Deleted: depth of each clusters has been indicated with le

Deleted: vels

Deleted: of

Deleted: at

Deleted:

Deleted: is shown

Deleted: in

Deleted: et

Deleted: nodes

Table 1. *Strains investigated.*

Strain	Origin	Farm/ rotation	Lesion	Identification by 16S rRNA and PFGE	Identification API20 STREP	PFGE cluster	Date
Strains investigated							
21192H4L3	Broiler parents	I/2	Septicaemia	<i>Enterococcus avium</i>	<i>E. avium</i>	A2	2005
21192H3L2 ^a	Broiler parents	I/2	Septicaemia	<i>E. avium</i>	<i>E. avium</i>	A2	2005
21007CHL1	Broiler chicken	NI ^b	Septicaemia	<i>E. faecium</i>	<i>E. faecium</i>	A3	NI
21147H3L3	Broiler parents	I/2	Endocarditis, septicaemia	<i>Streptococcus pluranimalium</i>	<i>S. bovis</i> II 3	A1	2005
21090H4L4	Broiler parents	I/2	Septicaemia	<i>S. pluranimalium</i>	<i>Aerococcus viridans</i> 1	A1	2005
21261H3L2	Broiler parents	I/2	Septicaemia	<i>S. pluranimalium</i>	<i>Aerococcus viridans</i> 1	A1	2005
21069H1L3	Broiler parents	I/2	Septicaemia	<i>S. pluranimalium</i>	<i>Leuconostoc</i> sp.	A1	2005
20978H4L2	Broiler parents	I/2	Endocarditis, septicaemia	<i>S. pluranimalium</i>	<i>Aerococcus viridans</i> 1	A1	2005
20978H4L6 ^a	Broiler parents	I/2	Endocarditis, septicaemia	<i>S. pluranimalium</i>	<i>Aerococcus viridans</i> 1	A1	2005
21231H1L4	Broiler parents	I/2	Salpingitis, peritonitis, endocarditis, septicaemia	<i>S. pluranimalium</i>	<i>Aerococcus viridans</i> 1	A1	2005
21192H2L2	Broiler parents	I/2	Endocarditis, septicaemia	<i>S. pluranimalium</i>	<i>Aerococcus viridans</i> 1	A1	2005
21248H2L2	Broiler parents	I/2	Endocarditis, septicaemia	<i>S. pluranimalium</i>	<i>Leuconostoc</i> sp.	A1	2005
21090H3L1	Broiler parents	I/2	Endocarditis, septicaemia, amyloidosis	<i>S. pluranimalium</i>	<i>S. salivarius</i>	A1	2005
21108H4L2	Broiler parents	I/2	Endocarditis, septicaemia	<i>S. pluranimalium</i>	<i>Aerococcus viridans</i> 1	A1	2005
21147H4L1	Broiler parents	I/2	Septicaemia	<i>S. pluranimalium</i>	<i>Leuconostoc</i> sp.	A1	2005
20315H1HJ4 ^a	Broiler parents	I/1	Endocarditis, septicaemia	<i>S. pluranimalium</i>	<i>A. urinae</i>	B2	2004
20363H2HJ3 ^a	Broiler parents	I/1	Endocarditis, septicaemia	<i>S. pluranimalium</i>	<i>Aerococcus viridans</i> 1	B2	2004
21147H4L2 ^a	Broiler parents	I/2	Septicaemia	<i>S. pluranimalium</i>	<i>S. salivarius</i>	B1	2005
21178H3L10	Broiler parents	I/2	Septicaemia, liver rupture	<i>S. pluranimalium</i>	<i>Aerococcus viridans</i> 1	B1	2005
21178H3L11	Broiler parents	I/2	Septicaemia	<i>S. pluranimalium</i>	<i>Aerococcus viridans</i> 1	B1	2005
21147H3L6 ^a	Broiler parents	I/2	Endocarditis, septicaemia	<i>S. pluranimalium</i>	<i>A. urinae</i>	B1	2005
20326H1L1 ^a	Broiler parents	I/1	Endocarditis, septicaemia	<i>S. pluranimalium</i>	<i>Leuconostoc</i> sp.	B1	2004
21129H3HJ3 ^a	Broiler parents	I/2	Endocarditis, septicaemia	<i>E. avium</i>	<i>E. avium</i>	B3	2005
20326H4L1 ^a	Broiler parents	I/1	Septicaemia	<i>Lactobacillus</i> sp.	<i>Gemella morbillorum</i>	B4	2004
Reference strains							
191 ^a	Broiler parents	II	Endocarditis, septicaemia	<i>S. pluranimalium</i>			2001
(= 16023H3L3C)							
C19672L28 ^a	Table-egg production	III	Septicaemia	<i>S. pluranimalium</i>			2004
178 (=178-9) ^a	Table-egg production	IV	Septicaemia	<i>S. pluranimalium</i>			2000
219 (=LiverLA3)	Broiler parents	II	Septicaemia	<i>S. pluranimalium</i>			1999

^a 16S rRNA gene sequence determined.^b NI, no information.

Table 2. Phenotypic characterization of *isolates of Streptococcus pluranimalium*, *Enterococcus avium* and *E. faecium*.

Characteristics	<i>Streptococcus</i> ^a <i>pluranimalium</i>	<i>Enterococcus avium</i> ^a	<i>E. faecium</i> ^a
Gram positive cocci in chains	+ ^b	-	(+)
α-haemolysis on bovine blood agar	+	w	+
Motility, 37 °C	-	-	-
Catalase	-	-	-
Oxidase, TMPD	-	-	-
Aerobic growth	+	+	+
Fermentative, Hugh & Leifson with glucose	+	+	+
Voges-Proskauer reaction	-	+	+
Hippurate hydrolysis	d (0.11)	-	+
Aesculine hydrolysis	+	+	+
Pyrrolidonyl arylamidase (PYR)	-	+	w
α-galactosidase	d (0.84)	-	+
β-glucuronidase	-	-	-
β-galactosidase	d (0.11)*	-	w
Alkaline phosphatase	d (0.17)*	-	-
Leucine aminopeptidase	+	+	+
Arginine dihydrolase	-	-	+
Production of acid from			
D-ribose	d (0.84)	+	+
L-arabinose	d (0.05)	+	+
D-mannitol	d (0.84)	+	-
D-sorbitol	d (0.79)	+	-
D-lactose	d (0.79)	+	+
D-trehalose	d (0.89)	+	+
Inulin	-	-	+
D-raffinose	d (0.74)	-	+
Starch	-	-	+
Glycogen	-	-	-
No. of isolates	19	3	1

^a Identification was evaluated by comparison of 16S rRNA sequences with type and reference strains and relationships derived from PFGE comparison.

^b +, 90% or more positive; -, 90% or more negative; d, different with proportion of positives in parenthesis; w, weak positive, 10-89% positive; *, weak positive reactions observed.

Deleted: ¶
Deleted: investigated
Deleted: The proportion of positives are given in parentheses
Deleted: .

Deleted: actual number of positives out of total
Deleted: 10-89% positive;
Deleted: ;
Deleted: positive
Deleted: for actual positives

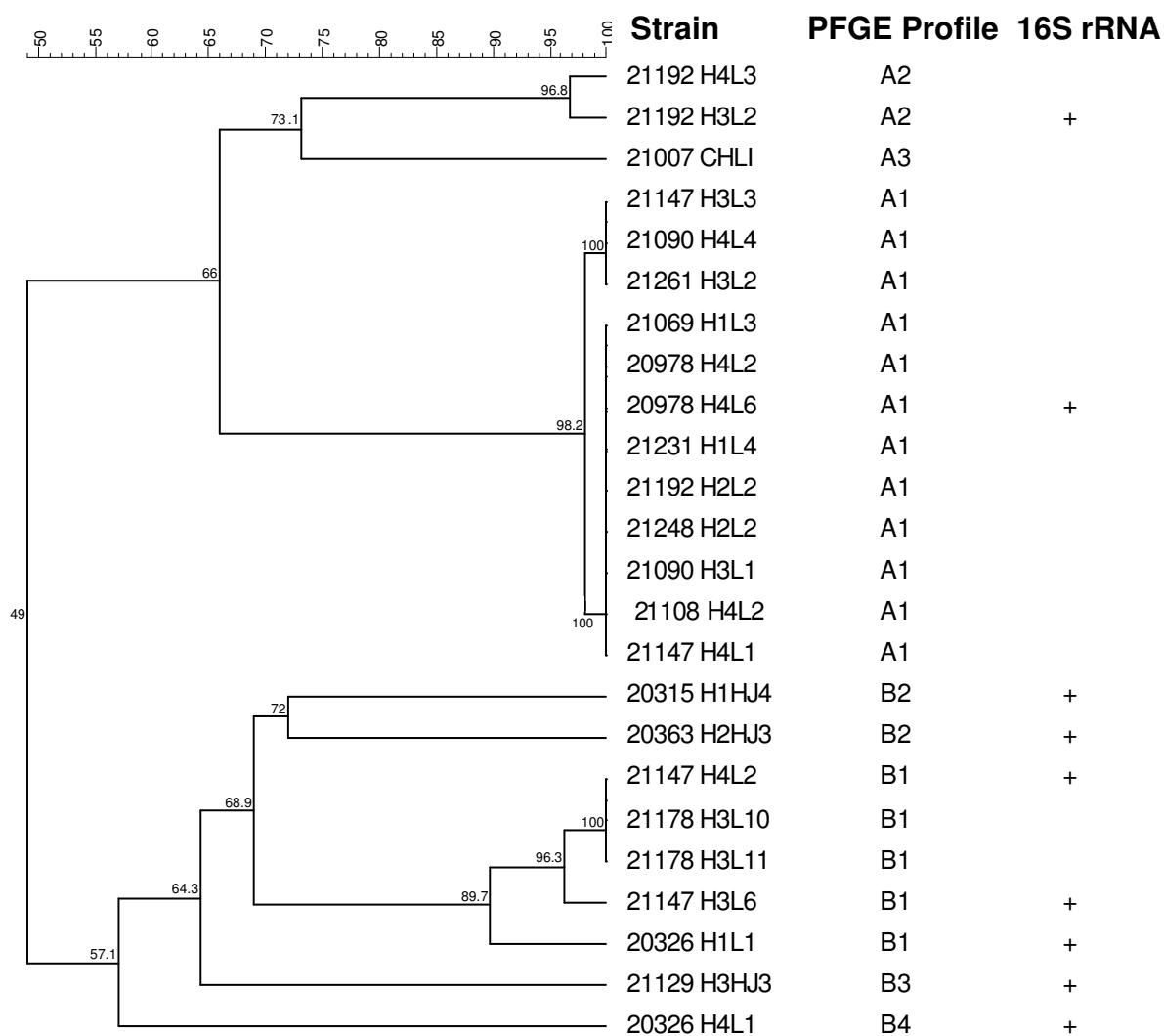


Fig. 2

