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**Clonality of *Enterococcus faecalis* associated with amyloid arthropathy in chickens evaluated by multi locus sequence typing (MLST)**

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49 **Abstract**

50

51 The aim of the study was to evaluate the clonality of 21 strains of *Enterococcus faecalis* associated with  
52 arthritis and amyloid arthropathy by multi locus sequence typing (MLST). The strains originated from  
53 five countries. Fifteen of the twenty-one strains demonstrated the same sequence type, ST82, including  
54 a reference amyloid arthropathy strain from the Netherlands. Some of the demonstrated sequence types  
55 (ST36, ST59 and ST82) have also been described from human clinical samples while other types are  
56 reported for the first time. The results confirm previous observations that outbreaks of amyloid  
57 arthropathy seem to be clonally related and indicate a wide distribution of the predominant sequence  
58 type which was demonstrated in four countries in Europe and in the USA.

59

60 Keywords: *Enterococcus faecalis*, amyloid arthropathy, MLST

## 61 Introduction

62

63 Amyloid arthropathy was first described in brown layers (Landman et al. 1994). Since then, uni, bi- or  
 64 polyarticular amyloid arthropathy, often accompanied by systemic amyloidosis, involving mainly the  
 65 liver and spleen, has been reported extensively in brown layers and less frequently in broiler parents  
 66 (Steentjes et al. 2002). It is often an unnoticed chronic disease with important economic impact. A  
 67 previous study has reported that up to 13% of an affected flock were culled (Petersen et al. 2008).  
 68 *Enterococcus faecalis* has been the most frequently demonstrated pathogen in cases of field outbreaks and  
 69 is considered to have a significant role in the pathogenesis (Landman et al. 1998). Natural outbreaks  
 70 associated with *E. faecalis* seem to be clonal or clonally related as demonstrated by pulsed-field gel  
 71 electrophoresis (PFGE) for strains from Netherlands and the USA (Steentjes et al. 2002). However,  
 72 comparison of PFGE profiles between different laboratories and between different runs within the  
 73 same laboratory may be difficult and dependent on subjective interpretation although international  
 74 collaborations between reference laboratories like PulseNet have standardised procedures for important  
 75 zoonotic and food-borne bacteria (Swaminathan et al. 2006). The population structure of many  
 76 veterinary important bacteria has been found to be clonal by multilocus enzyme electrophoresis, MLEE  
 77 (Selander et al. 1986; Blackall et al. 1998). Multilocus sequence typing (MLST) has now almost replaced  
 78 MLEE (Harbottle et al. 2006; Litrup et al. 2007). By MLST, the genes encoding enzymes involved in  
 79 conserved metabolic and information processes are compared at the DNA-sequence level (Maiden et  
 80 al. 1998). *E. faecalis* strains of human clinical and porcine origin have previously been examined by  
 81 MLST (Ruiz-Garbajosa et al. 2006; Shankar et al. 2006; Kawalec et al. 2007). In this work, *E. faecalis*  
 82 obtained from amyloid arthropathy in chickens from different countries were typed with MLST. This  
 83 represents to our knowledge one of the first uses of MLST for studying clonality among *E. faecalis* from  
 84 poultry.

## Materials and methods

A total of 21 strains were compared in the study (Table 1). Five strains originated from unrelated brown layers farms in France. Five strains were from layers in Germany, obtained over a time period of nine years. Strains were obtained from joints demonstrating amyloid deposits. One American reference strain was obtained from the laboratory of Dr. W. Landman. The sequence types (ST) of nine Danish strains and one Dutch reference strains, all obtained from chicken with amyloid arthropathy, have recently been published (Petersen et al. 2008). Information regarding these strains has also been included in Table 1.

MLST was performed based on the scheme published at Multi Locus Sequence Typing home page, using the primers and PCR conditions as described (<http://efaecalis.mlst.net/>). The exception was the primers published for the *gyd* gene. It was not possible for us to generate a sequence from the PCR product. Instead, a new set of primers was designed, *gydFnew* 5'-CAAACCATGAAACATTAAGTGG-3' and *gydRnew* 5'-AAGTTAGCGAAGTATTCTAAAGTACGA-3' with an expected amplicon size of 597 bp and otherwise unchanged PCR conditions. Amplification products were sequenced in both directions using the PCR primers at Macrogen Inc., Seoul, Korea. The obtained sequences were compared to published alleles and a ST was assigned to each strain (<http://efaecalis.mlst.net/>). To illustrate the evolutionary relatedness of *E. faecalis*, a population snapshot was made of all published STs using the eBURST programme available at <http://efaecalis.mlst.net/>. Sequences and strain information have been deposited in the *E. faecalis* MLST database at the same web site.

## 106 Results and discussion

107

108 Seven out of eleven strains were in this study found to be MLST type ST82 (Table 1). This is the same  
 109 ST as the Dutch reference strain 6085.94 (Petersen et al. 2008). ST82 was also recently demonstrated as  
 110 the predominant ST among *E. faecalis* associated with amyloid arthropathy in Denmark (Petersen et al.  
 111 2008). In addition, two Danish strains were ST170 that is closely related to ST82 (six out of the seven  
 112 alleles were identical; *xpt* is allele 1 in ST170 and allele 10 in ST82 (Petersen et al. 2008)). ST82 was first  
 113 described in a strain originating from a blood sample of a hospitalised patient in Poland (Kawalec et al.  
 114 2007). One strain (K100/2003) from Germany was ST36. This sequence type was first isolated from a  
 115 human clinical blood sample in Spain (Ruiz-Garbajosa et al. 2006). A French strain (K1233/04) was  
 116 ST59, a sequence type first demonstrated in a faeces sample from a healthy pig and subsequently  
 117 obtained from hospitalised patients in Poland (Ruiz-Garbajosa et al. 2006; Kawalec et al. 2007). ST59  
 118 shares six of seven alleles with ST33 in the MLST database, a clinical strain obtained from a human  
 119 blood sample (Ruiz-Garbajosa et al. 2006).

120

121 *E. faecalis* demonstrates major genetic diversity (McBride et al. 2007). However, most strains with a  
 122 confirmed association with amyloid arthropathy were highly clonal as demonstrated by the MLST. Six  
 123 out of ten French and German strains demonstrated the same sequence type, ST82. In particular, four  
 124 out of five German strains demonstrated this ST and these strains were isolated over nine years. In  
 125 addition, nine previously described Danish outbreak strains (Petersen et al. 2008) and the Dutch and  
 126 American reference strains were ST82 (n=9) or closely related ST170 (n=2). Possible explanations for  
 127 the wide distribution of a single clone include vertical transmission, trading of live poultry and  
 128 horizontal transmission through humans or migratory birds. In addition, a Dutch study suggested a  
 129 possible association with Marek's disease vaccination through contaminated injection needles and  
 130 vaccine suspensions (Landman et al. 2000).

131

132 A population snapshot of all recognised STs of *E. faecalis* is displayed in Figure 1. The snapshot  
 133 confirms the genetic diversity and demonstrates that the STs associated with amyloid arthropathy in  
 134 chicken (encircled in Figure 1) do not belong to any of the recognised clonal complexes (CC). Until  
 135 now, only few strains of chicken origin exist in the MLST database, but they are all closely related to  
 136 strains of human origin. One strain obtained from an unspecified chicken product was ST5 which  
 137 belongs to CC21. This clonal complex consists of strains mostly of human clinical origin. Another  
 138 strain obtained from chicken faeces was ST 32, which is identical in 6 alleles to ST4, a human clinical  
 139 strain (ST information from [efaecalis.mlst.net](http://efaecalis.mlst.net)). Thus, the observation in this study of an association  
 140 between human clinical and chicken strains supports previous findings.

141

142 The use of MLST enabled an unambiguous typing of the strains. MLST is a definitive and sensitive  
 143 typing method (Aanensen and Spratt 2005). The *E. faecalis* scheme was developed to study the clonality  
 144 of human clinical strains (Ruiz-Garbajosa et al. 2006) and have also been used in a study of strains from  
 145 pigs (Shankar et al. 2006). In this study MLST was successfully used to establish clonality and  
 146 evolutionary relationships between amyloid arthropathy causing strains. It will be a useful tool to track  
 147 and control diseases in poultry globally, including potential zoonotic clones. The typing revealed that  
 148 disease-causing *E. faecalis* ST82 apparently does not have one specific host but is able to cause  
 149 infections in both humans and chickens. Further investigations are, however, needed to clarify if ST82  
 150 represents a genuine zoonoses or occurs as a normal inhabitant of the human gastro-intestinal tract. So  
 151 far ST82 has not been associated with other lesions in poultry than amyloid arthropathy and nothing is  
 152 known as to its prevalence in normal chickens of different genetic lines.

153

154 Amyloid arthropathy often goes unnoticed in the initial phase and may subsequently develop into a  
 155 chronic disease, resulting in increased cullings and uneven flocks (Petersen et al. 2008). The disease may



156 have an important economic impact and further studies are required to elucidate virulence properties of  
157 the infectious agents, treatment and prevention strategies.

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159

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212

213

214 **Figure legend:**

215 Figure 1. Population snapshot of all sequence types (ST) included in the MLST database for *Enterococcus*  
216 *faecalis*. The snapshot was made using the eBURST programme. Evolutionary related STs, sharing six  
217 out of seven alleles, are connected by lines. The STs associated with amyloid arthropathy in chicken  
218 have been encircled.

219

Table 1

*Enterococcus faecalis* strains isolated from amyloid arthropathy included in the study

Strain/isolate	Origin	Year	Country	Sequence type (ST) <sup>a</sup>	Allelic profile <sup>b</sup>	Reference
<i>ST determined in this study</i>						
K923/96	Brown layer	1996	Germany	82	12,6,28,29,7,10,20	This study
K37/2003	Brown layer	2003	Germany	82		This study
K100/2003	Brown layer	2003	Germany	36	16,2,19,16,17,15,11	This study
K485/2005	Brown layer	2005	Germany	82		This study
K512/2005	Brown layer	2005	Germany	82		This study
K607/04-1	Brown layer	2004	France	82		This study
K607/04-3	Brown layer	2004	France	82		This study
K607/04-4	Brown layer	2004	France	171	12,6,7,5,7,4,1	This study
K1082/04	Brown layer	2004	France	172	8,7,28,29,7,4,20	This study
K1233/04	Brown layer	2004	France	59	14,2,18,10,16,2,12	This study
042000-03106	Brown layer	no information	USA	82		Steentjes <i>et al</i> 2002
<i>Strains with previously determined ST (Petersen et al 2008)</i>						
6085.94	Brown layer	1994	Netherlands	82		Landman <i>et al</i> 1994
C19315/Led 1A	Lohmann silver layer	2004	Denmark	82		Petersen <i>et al</i> 2008
C19315/Led 2A	Lohmann silver layer	2004	Denmark	82		Petersen <i>et al</i> 2008
C19315/Led 4A	Lohmann silver layer	2004	Denmark	82		Petersen <i>et al</i> 2008
C19315/Led 5A	Lohmann silver layer	2004	Denmark	170	12,6,28,29,7,1,20	Petersen <i>et al</i> 2008
C19363/4 led 2	Lohmann silver layer	2004	Denmark	170		Petersen <i>et al</i> 2008
C19363-11/Led 1	Lohmann silver layer	2004	Denmark	82		Petersen <i>et al</i> 2008
C19445/Led 1PP	Lohmann silver layer	2004	Denmark	82		Petersen <i>et al</i> 2008
OTB/Led 13	Lohmann silver layer	2004	Denmark	82		Petersen <i>et al</i> 2008
SC 3	Lohmann silver layer	2004	Denmark	82		Petersen <i>et al</i> 2008

a) As determined by multi locus sequence typing

b) Allele no for *gdh*, *gyd*, *pstS*, *gki*, *aroE*, *xpt* and *yqiL*, respectively. Only once for each ST.

