Anellovirus is a recently created, floating genus of small, non-enveloped viruses that infect a wide range of mammalian species (Biagini et al., 2004). Their genome is composed of circular, negative-sense, single-stranded DNA that carries an untranslated region (UTR) and at least two major ORFs. The type species, *Torque teno virus* (TTV), was discovered in a human patient affected by hepatitis of unknown origin (Nishizawa et al., 1997). In a short time, the suspected relationship between TTV and hepatitis, or any other pathology, was rejected after multiple reports of very high viral prevalences in diverse human populations, ranging around 80% (reviewed by Bendinelli et al., 2001). Moreover, TTV infects not only the liver, but also many other organs, of a single individual without apparent pathology. Between isolates, the virus exhibited high genomic diversity: at the time of writing, human TTV variants are classified into five major phylogenetic groups (Peng et al., 2002). Noticeably, human individuals may carry several distinct genotypes (Ball et al., 1999; Biagini et al., 1999; Jelcic et al., 2004; Kakkola et al., 2004; Verschoor et al., 1999).

Other anelloviruses have been partially or fully characterized in several animal species, including domestic animals, such as dogs, cats, pigs, bovines, chickens and ovines, as well as wild animals, such as tupaias (tree shrews) and non-human primates (Cong et al., 2000; Leary et al., 1999; Okamoto et al., 2001b, 2002; Verschoor et al., 1999). Interestingly, the genome of anelloviruses tends to be smaller when the order of the infected animal is lower, varying from 3.8 kb for human viruses to 2.2 kb for tupaias and reaching 2.9 kb for the only available full-length viral genome from a pig (*Sus domesticus*) in Japan (tentatively named TTV-Sd31) (Okamoto et al., 2002). Although less exhaustive than for humans, studies suggest high natural prevalences of anelloviruses in some animals, as well as some intra- and inter-individual genetic variability (Okamoto et al., 2001b; Thom et al., 2003; Verschoor et al., 1999). Recently, viral prevalences ranging between 33 and 100% have been reported in the sera of pigs from Canada, China, Korea, Spain, Thailand and the USA (McKeown et al., 2004).

Here, we report the first molecular survey of pig anelloviruses in herds in Brittany (France). This study was conducted in order to estimate the prevalence and diversity of the virus genetic pool and its associated risks. Initially, samples were collected in pig herds all over Brittany, France, between July 2000 and January 2002, for an epidemiological study of another member of the family *Circoviridae*, *Porcine circovirus 2* (PCV2) (de Boisséson et al., 2004). Among the available samples, a series was chosen randomly for the search for anelloviruses. From 14 herds [named H-t and v (de Boisséson et al., 2004)], one to three animals (total 32, named with a Greek letter) were analysed for virus presence in five tissue samples (tonsil, ileum, lung and inguinal and mesenteric lymph nodes). In an additional herd (herd w), a single organ (lung) was
collected from a piglet that was killed in 2003. Tissues were collected and stored at \(-20^\circ C\) until DNA extraction and PCR analysis.

Total DNA was extracted by using a DNeasy tissue kit (Qiagen) from 20 mg frozen tissue. DNA was resuspended in 20 \(\mu\)l buffer. For PCR, the two primers COM-1sens (5'-CRSWKMCAGATGGYWAGTTTWY-3') and COM-2rev (5'-GCCCGAAATTGCCCTTWGACTKCG-3'), which targeted two conserved domains within the UTR, were used (Biagini et al., 2003). A 2 \(\mu\)l aliquot of DNA was used in a reaction volume of 50 \(\mu\)l containing 90 pmol each primer, 1.5 mM MgCl\(_2\) and 2 U Taq polymerase (Eurobio). An initial step of 4 min at 94 \(^\circ C\) was performed, followed by 38 cycles of 94 \(^\circ C\) for 1 min, 57 \(^\circ C\) for 30 s, 72 \(^\circ C\) for 20 s and a final elongation step of 5 min at 72 \(^\circ C\). A 20 \(\mu\)l aliquot of the reaction product was electrophoresed on a 3 % TAE/agarose gel stained with ethidium bromide. DNA bands of the expected size (about 115 bp) were gel-purified and TA-cloned in pCR4-TOPO (Invitrogen). Sequencing was performed on both strands by using an ABI Prism dye terminator cycle sequencing kit and an ABI Prism 3100 Avant sequence analyser (Perkin Elmer). Between two and ten bacterial clones were sequenced for each cloned PCR product. Series of negative controls (water) were performed repeatedly for each amplification, to ensure the absence of contamination.

Nucleotide sequences were edited with the package Vector NTI Advance (Informax) and aligned with the CLUSTAL W method implemented in the package. Identity levels were obtained by simultaneously aligning all of the representative sequences produced here and anellovirus sequences from other isolates available in GenBank. Phylogenetic analysis of aligned sequences was performed by using the neighbour-joining method with the Tamura–Nei \(\gamma\) model, implemented in the MEGA3 program (Kumar et al., 2004; available at http://www.megasoftware.net/mega3/). A bootstrap resampling analysis of 500 replicates was performed.

Anelloviruses were detected in 93 % (14/15) of the herds. Only herd r, in which two animals were tested, was negative. The frequency of animals that were positive for at least one organ was 73 % (24/33). When an individual was found to be positive, not all of its organs necessarily gave a PCR product. For instance, in herds h–w, only one animal was positive for all five organs, whereas about one-third (10/32) of the animals had one positive organ (three positive organs, 21.9 %; four organs, 9.3 %; two organs, 6.2 %). In total, 32.5 % (52/160) of all tested organs were positive, the lung being the most frequently positive organ (lung, 11.2 %; inguinal lymph node, 8.1 %; mesenteric lymph node, 5.6 %; tonsil, 4.4 %; ileum, 3.1 %).

For each animal that exhibited at least one positive organ, one PCR product was cloned and a few (two to ten) clones were sequenced. In total, 82 sequences were obtained that, once aligned, were grouped in a total of 24 variants that exhibited between 78 and 99 % identity to one another. The sequences varied between 59 and 78 bp, differing by point mutations combined (or not) with insertions/deletions (Fig. 1). However, it cannot be excluded that...

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**Fig. 1.** Nucleotide sequence alignment of anellovirus variants obtained from Brittany, France. The alignment was performed by using CLUSTAL W (see text) with additional, minor manual adjustments. The nomenclature mainly follows the latest recommendations of the International Committee on Taxonomy of Viruses (Biagini et al., 2004). In addition, the region of origin of clones is shown in the name (FR, France; JP, Japan), the herd of origin is indicated in lower case (h–w) and the name of the animal is given as a Greek letter. Two unique variants (bold) from Japan were added for comparison. Grey blocks indicate well-conserved residues; dark blocks indicate fully conserved residues.
some variants, unique by one single base, may have arisen
from PCR artefacts, despite the small size of the amplicon.
All of our viral clones showed the motif GMCTGGG-
CGGGTGCCGVA in a region that is conserved among
anelloviruses. Two main clades, A and B, were distinguished
in the phylogenetic tree (Fig. 2). Branch B includes all of
the clones with the shortest sequences, as well as several
clones of 73 bp. Considering that the UTR region is well-
conserved among anelloviruses, more differences are
expected in comparison of the full-length genomes.

Sequence heterogeneity within an isolate is common
among anelloviruses. As expected, heterogeneity was pres-
ent in some of our isolates. By comparing clones from
different organs of a single animal, variability was readily
observed in two animals by obtaining two amplicons of
very similar, yet distinct, sizes on a 3 % agarose gel (data
not shown). For one animal in herd i, the lung exhibited a
long product and the inguinal lymph node a short pro-
duct. In a second case (herd j), amplicons of both sizes
were produced from the lung, whereas the inguinal lymph
node gave only the long product. In both cases, sequencing
confirmed the existence of two populations of clones
differing by 10 nt, each population clustering separately in
branches A and B of the phylogenetic tree. The differences
were not limited to a set of short deletions in the popula-
tion from branch B, but also to several point mutations
that were conserved in each subgroup, suggesting the
coexistence of at least two distinct subtypes, not simply
derived from one another by a single mutation event.

Intra-organ variability was further evidenced by the
sequences of 10 clones that were obtained from the lung
of an animal from herd w. Six were strictly identical and
grouped in branch B, whereas the other four, almost
identical to one another, grouped in branch A, sharing
93 % identity with the six sequences of branch B (Fig. 2).

The lowest level of identity among our isolates was reached
when comparing clones from two herds separated by
150 km (n and j) (Table 1). However, this level (78 %) was
in the same range as the lowest level reached by two clones
originating from the same herd, e.g. in herd i (81 %). This
suggests that, although limited in number, our samples
represent the global viral diversity in the region quite well.

To evaluate the relationship of our isolates with other
anelloviruses, our variants were aligned with comparable
DNA domains of anelloviruses from various hosts, includ-
ing seven sequences of swine anelloviruses from Japan,
i.e. the only full-length (renamed here Sd- TTV-JP31) and
six partial sequences of other isolates. The identity levels
between any of our clones and sequences from other hosts
varied from 67 to 82 % (Table 1), the sequences from
the owl monkey and tupaia being the least and the most
similar, respectively. It is to be noted that some of our
clones, when compared, reached levels of identity that
were similar to or slightly lower than those to sequences of
tupaia. However, the phylogenetic analysis performed after
alignment showed a complete separation, not supported by
bootstrap analysis, of our clones from the viruses of other
hosts, whilst grouping together all the sequences from swine

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**Fig. 2.** Phylogenetic tree of the equivalent UTR domain of anel-
loviruses from swine and other hosts. Clones with identical
sequences are included in the branches. The number of identi-
cal clones found within a given herd is indicated in parenth-
eses. For clarity, the identification of the individuals carrying
the viral clones is not indicated. The tree includes sequences origi-
nating from dog (TTV10-Cf, TTV16-Cf and TTV9-Cf), cat
(TTV2-Fc, TTV7-Fc and TTV4-Fc), human (TTV-TYM9,
TTV-TUS01, TTV-L01, TTV-TA278 and TTV-yonKC009), owl
monkey (TTV3-Ai), macaca (TTV-Mf-TTV9 and TTV3-Mf), tupaia
(TTV14-Tbc) and pig (GenBank accession numbers in parenth-
eses)

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**Table 1.** Similarity levels between sequences from different
hosts.

<table>
<thead>
<tr>
<th>Host</th>
<th>Accession Numbers</th>
<th>Identity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dog</td>
<td>TTV10-Cf, TTV16-Cf, TTV9-Cf</td>
<td>78</td>
</tr>
<tr>
<td>Cat</td>
<td>TTV2-Fc, TTV7-Fc, TTV4-Fc</td>
<td>75</td>
</tr>
<tr>
<td>Human</td>
<td>TTV-TYM9, TTV-TUS01, TTV-L01, TTV-TA278, TTV-yonKC009</td>
<td>70</td>
</tr>
<tr>
<td>Owl monkey</td>
<td>TTV3-Ai</td>
<td>69</td>
</tr>
<tr>
<td>Macaca</td>
<td>TTV-Mf-TTV9, TTV3-Mf</td>
<td>68</td>
</tr>
<tr>
<td>Tupaia</td>
<td>TTV14-Tbc</td>
<td>67</td>
</tr>
<tr>
<td>Pig</td>
<td>GenBank accession numbers in parentheses</td>
<td>67-82%</td>
</tr>
</tbody>
</table>
(French or Japanese) (Fig. 2). Noticeably, some clones from Brittany were identical to several Japanese sequences (Fig. 2). This complete identity was observed for clones from clusters A and B, indicating that these Japanese isolates followed the separation in at least two distinct populations with respect to this UTR domain.

Based on a short sequence from a conserved domain, we showed that our sequences are related genetically to Sd-TTV31 and other Japanese isolates, with at least two subpopulations that are not restricted geographically, but mixed. Such a distribution suggests a common pool of genomes disseminated in the two regions, via an as-yet-unknown route. Hypothetically, dissemination could be amplified within an herd or a region by air-borne transmission, considering that the lung is the most frequently positive organ among those tested. The lung is also a site of virus accumulation in human beings (Okamoto et al., 2001a).

Whether swine anelloviruses represent a sanitary risk to their natural host or other hosts remains unknown. In our survey, most animals were healthy, although they were carriers of PCV2, supporting the hypothesis that the anellovirus isolates are not pathogenic in swine. However, the situation of anelloviruses in swine is reminiscent of the high worldwide prevalence of PCV2. This virus, which has coexisted with swine for a long time, has been associated with a recently emerged pathology, post-weaning multisystemic wasting syndrome, probably as a consequence of an as-yet-unidentified biological factor or a modern breeding practice. It appears that, in the same samples from Brittany, the diversity of anelloviruses is superior to that of PCV2, which exhibits a high degree of conservation in Brittany (de Boisséson et al., 2004). Despite the high host specificity demonstrated by anelloviruses to date and their non- or poor viability in heterologous hosts (Mushahwar et al., 1999; Okamoto et al., 2000), frequent exposure of human beings to a potentially heterogeneous inoculum produced by herds of numerous virus carriers is a sanitary risk that must not be neglected.

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References


