Assessment of collagen genes involved in fragmented medial coronoid process development in Labrador Retrievers as determined by affected sibling-pair analysis

Katja G. Salg, DVM; Jedee Temwitchitr, DVM; Sandra Imholz; Herman A. W. Hazewinkel, DVM, PhD; Peter A. J. Leegwater, PhD

Objective—To evaluate the involvement of various collagen genes in the development of fragmented medial coronoid process (FCP) in Labrador Retrievers.

Sample Population—93 dogs originating from 13 litters were used in the study; FCP was diagnosed in 35 dogs, and each affected dog had at least 1 sibling that was also affected. Twelve dams and sires were included in the analysis. All dogs were purebred Labrador Retrievers except for 2 litters (offspring of a female Golden Retriever-Labrador Retriever mixed-breed dog).

Procedures—For each dog, DNA was isolated from blood samples. Polymorphic microsatellite markers adjacent to 14 candidate genes (ie, COL1A1, COL1A2, COL2A1, COL3A1, COL5A1, COL5A2, COL6A3, COL9A1, COL9A2, COL9A3, COL10A1, COL11A1, COL11A2, and COL24A1) were analyzed by use of PCR assays; genotypes were determined via automated detection of DNA products. The level of allele sharing between pairs of affected siblings was assessed.

Results—Among the 93 dogs, allele sharing of the 14 collagen genes was determined as follows: COL1A1, 45%; COL1A2, 47%; COL2A1, 37%; COL3A1, 32%; COL5A1, 43%; COL5A2, 32%; COL6A3, 36%; COL9A1, 45%; COL9A2, 49%; COL9A3, 38%; COL10A1, 46%; COL11A1, 52%; COL11A2, 47%; and COL24A1, 47%.

Conclusions and Clinical Relevance—Because siblings share 50% of their genome at random, the fact that the percentages of allele sharing among the analyzed collagen genes were not significantly > 50% indicates that these genes are not determinant candidates for FCP in Labrador Retrievers. The gene for the vitamin D receptor could also be excluded because of its proximity to COL2A1. (Am J Vet Res 2006;67:1713–1718)

Elbow joint dysplasia is the most common heritable disease causing forelimb lameness in dogs. Elbow joint dysplasia comprises different types of growth disorders including ununited anconeal process, OCD, FCP, and incongruity of the elbow joint. Fragmented medial coronoid process is a condition that is prevalent in several dog breeds such as Rottweiler, German Shepherd Dog, Golden Retriever, Bernese Mountain Dog, and Labrador Retriever. In Labrador Retrievers, the first clinical signs such as elbow-related lameness and signs of pain are detected between 4 and 8 months of age. Although osteoarthritis can be diagnosed radiographically, an FCP is not always detectable. Visualization of the FCP often warrants additional imaging techniques or arthrotomy, the latter as part of surgical treatment. Although FCP has been described as an inherited disease, little is known about the inheritance pattern. Ubbink et al determined that Labrador Retrievers from groups of FCP-affected dogs are more related to each other than Labrador Retrievers from groups of unaffected dogs. Those investigators were able to trace back the trait to a limited number of founders from a large group of contemporary breeding dogs and could predict the incidence of FCP among dogs on the basis of their relationship to these founders. However, this analysis could not reveal the mode of inheritance.

Results of 2 studies indicated that that OCD and FCP are inherited as independent traits. For instance, in their study, Padgett et al determined that OCD and FCP did not cosegregate in a cross and backcrosses of Labrador Retrievers affected by both traits. Another study revealed that FCP and incongruity of the elbow joint are independently inherited diseases in Bernese Mountain Dogs. Findings of several studies have suggested that inheritance for OCD and FCP is neither recessive nor sex linked. The reported heritability (h²) varies from 0.27 to 0.77. Results of a previous study indicated that a major gene is associated with development of FCP, but a polygenic inheritance pattern has also been suggested. Also, environmental factors such as nutrition and exercise probably influence the development of FCP.

Among Labrador Retrievers, FCP is more prevalent in males than in females. The ratio of affected

ABBREVIATIONS

OCD Osteochondritis dissecans of the medial humeral condyle
FCP Fragmented medial coronoid process
RH Radiation hybrid
lod Logarithm of the odds (to the base 10)
NPL Nonparametric linkage

Received March 8, 2006.
Accepted April 23, 2006.
From the Department of Clinical Sciences of Companion Animals, Faculty of Veterinary Medicine, Utrecht University, PO Box 80154, 3508 TD Utrecht, The Netherlands. Supported by the Royal Dutch Foundation for Guide Dogs for the Blind (KNGF-Geleidehonden) and Hill’s Pet Nutrition. Address correspondence to Dr. Leegwater.
males to females varied from 2.1 to 5:1 in different studies. For FCP, general failure in endochondral ossification of the ulna is suspected to be the causative abnormality; although mechanical overloading of the medial coronoid process cannot be ruled out. In humans, a major part of bone dysplasia disorders is attributable to mutations in genes that code for collagen proteins.

The purpose of the study reported here was to evaluate the involvement of various collagen genes that are associated with bone dysplasias in humans in the development of FCPs in Labrador Retrievers. The gene codings for collagen type I α-1 (COL1A1), COL1A2, COL2A1, COL3A1, COL5A1, COL5A2, COL6A3, COL9A1, COL9A2, COL9A3, COL10A1, COL11A1, COL11A2, and COL24A1 were selected as candidate genes. All these candidate genes are involved in bone disorders as osteogenesis imperfecta, Stickler syndrome, or osteoarthritis in humans.

Materials and Methods

Sample collection—Blood samples (8 mL) were obtained specifically for genetic studies from 93 Labrador Retrievers born in 1989 through 1999. All dogs were part of a breeding program of the Royal Dutch Foundation for Guide Dogs for the Blind and were raised under identical nutritional regimens and conditions. Part of this population has been described previously. At the age of 12 to 18 months, all dogs had been examined for FCP by use of an extensive clinical and radiographic protocol. The disease status in all affected dogs had been confirmed by arthrotomy. Only litters with at least 2 affected siblings were used in the genetic analysis (Figure 1). The study group comprised 35 affected siblings, 46 unaffected siblings, and 12 dams and sires. The dams and sires were free of FCP except for the sire of pedigree B, in which FCP was diagnosed at a later age. A sample of DNA from this dog was not available.

The dogs were purebred Labrador Retrievers, except for 2 litters of a single dam who was a 1:1 mix of Labrador Retriever and Golden Retriever. Genomic DNA was isolated from blood leukocytes by use of the salt extraction method and frozen at −20°C until used. This study was performed with consent of the owners.

RH mapping—The COL11A1 and COL11A2 genes were localized on the canine genome with the RH panel RHDF5000. The human coding sequence of COL11A1 (GenBank accession No. NM_000093) and the genomic region containing exons 61 and 62 of COL11A2 (U41068) were compared with primary DNA data from the canine genome sequence project. The primary data were accessed through the trace archives of the National Center for Biotechnology Information, and the comparison was made with the conserved basic local alignment search tool (BLAST) option. Parts of the genes were reconstituted by assembling the primary data that had high similarity to the human DNA sequences into a contiguous sequence of DNA. Oligonucleotides (primers) for amplification of specific DNA fragments were designed on the basis of these reconstituted DNA sequences. The oligonucleotide sequences for amplification of the COL11A1 fragment were 5'-TGTCCCTCGTCAC-AATTGG-3' and 5'-GGACCACTGGGTCA-TGTCA-3'. The oligonucleotide sequences for amplification of the COL11A2 fragment were 5'-CAGACGTGTCGTGAGAT-3' and 5'-TGCTGTATGTCCTCCACCAA-3'.

A PCR procedure with a temperature gradient for the annealing step was performed on genomic canine DNA, hamster DNA, and a 2:1 mixture of hamster and canine DNA to optimize the PCR specificity for canine DNA. Fragments of DNA were amplified in a thermal cycler in a volume of 15 μL with 1X PCR buffer, 200μM dNTPs, 1.5mM MgCl₂, 0.8mM primers, 0.6 units of platinum Taq polymerase, and 25 ng of DNA. The PCR reaction comprised 10 minutes at 95°C, 35 cycles of 30 seconds at 94°C, 30 seconds at the optimized temperature, and 1 minute at 72°C; to complete the reaction, a step of 2 minutes at 72°C was performed. The reaction products were stored at 4°C.

The PCR products were analyzed on a 1% agarose-gel, and the presence or absence of a copy of the canine gene was assessed for each cell line of the RH panel. The outcome was compared by use of a computer program for RH mapping with the outcome of all available markers at the Centre National de la Recherche Scientifique (CNRS) of the University of Rennes, France, to place the genes on the CNRS RHDF5000 map of the dog genome.

Genotyping of microsatellite markers—Polymorphic microsatellite markers were selected from the RHDF5000 map for COL1A1, COL1A2, COL2A1, COL11A1, and COL11A2 (Appendix). The selected markers had a high level of heterozygosity or a location close to the gene. The PCR reactions (15 μL) contained 25 ng of genomic DNA, 0.33μM of forward primer, 0.33μM of reverse primer, 2.5mM MgCl₂, 200μM dNTPs, PCR gold buffer, and 0.3 units of a DNA polymerase. One of the primers was labeled by the manufacturer at the 5’ end with a fluorophore. Several collagen genes were localized by nucleotide-nucleotide BLAST (BLASTN) searches of the completed canine genome DNA sequence at the National Center for Biotechnology Information. The human reference complementary DNA sequences for COL3A1 (NM_000090), COL5A1 (NM_000093), COL5A2 (NM_000093), COL6A3
with 6-FAM, 25 mM MgCl2, 1 mM dNTPs, PCR gold buffer, DNA, and 10 μM reverse primer, 10 μM M13-tailed forward primer labeled at the 5′ end with 6-FAM, 25 mM MgCl2, 1 mM dNTPs, PCR gold buffer, and 0.3 units of a DNA polymerase.

Thermal cycling was carried out in a thermal cycler with a program as follows: 5 minutes at 95°C, followed by 10 cycles of 30 seconds at 95°C, 15 seconds at the annealing temperature, and 30 seconds at 72°C, then another 25 cycles of 30 seconds at 92°C, 15 seconds at the annealing temperature, and 30 seconds at 72°C. The program was completed with a step of 10 minutes at 72°C.

The PCR reactions were diluted 10- to 30-fold with H2O, and 2 μL of the dilution was mixed with 10 μL of formamide and 0.2 μL of fluorophore-labeled size standard. The products were analyzed by use of an automated DNA sequencer. The DNA products were sized and alleles were assigned by use of specialized software.

Linkage analysis—A sibling-pair linkage analysis was performed with the genotypes of FCP-affected siblings, unaffected siblings, and dams and sires when available. The allele sharing of each affected sibling pair was evaluated. Haplotypes of consecutive markers in each region with a candidate gene were constructed. Only data of informative siblings and dams and sires were taken into account; this means that only haplotypes were included in the calculations if the parental origin of the haplotype was certain and apparent recombinant offspring was discarded. The sum of all haplotypes shared by each pair of affected siblings was divided by the mean expected level of 50%. For all analyses, a value of P < 0.01 was considered significant.

Results

The chromosomal localizations of the collagen genes COL11A1 and COL11A2 were determined via RH mapping with the RHDF5000 panel. The outcome of the PCR reaction for COL11A1 was compared with the outcomes of other markers on the RHDF map. The lod score for COL11A1 was 13.1 and 11.0 with the markers FH3246 and EST17G5, respectively, of RHDF5000. These markers are located on CFA06 in a region that is syntenic with human chromosome 1p21, which also contains COL11A1.

The PCR results for the canine COL11A2 gene resulted in a lod score of 16.0 and 15.4 with the markers DLADVQ and EST2A7, respectively. This result was also in agreement with the expected localization because these markers are located on CFA12 in a region that is syntenic with human chromosome 6p21, which also contains COL11A2. The positions of COL11A1 and COL11A2 were derived on the basis of the markers that had the maximum lod scores (Appendix).

We investigated the involvement of a variety of collagen genes in development of FCP in Labrador Retrievers by measurement of allele sharing of nearby located microsatellite markers between affected litters. In general, the genes COL1A1, COL1A2, COL2A1, COL3A1, COL5A1, COL5A2, COL6A3, COL9A1, COL9A2, COL9A3, COL10A1, COL11A1, COL11A2, and COL24A1 were not associated with significantly high allele sharing (Table 1). None of the markers analyzed resulted in high allele sharing and a high NPL score with a significantly low P value. The highest NPL score of 0.56 was obtained for COL11A1.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Allele sharing (%)</th>
<th>NPL score</th>
<th>P value</th>
<th>Information content*</th>
</tr>
</thead>
<tbody>
<tr>
<td>COL1A1</td>
<td>45</td>
<td>0.4690</td>
<td>0.672</td>
<td>0.54</td>
</tr>
<tr>
<td>COL1A2</td>
<td>47</td>
<td>-0.0854</td>
<td>0.522</td>
<td>0.66</td>
</tr>
<tr>
<td>COL2A1</td>
<td>37</td>
<td>-0.2317</td>
<td>0.581</td>
<td>0.57</td>
</tr>
<tr>
<td>COL5A</td>
<td>43</td>
<td>-0.1969</td>
<td>0.566</td>
<td>0.40</td>
</tr>
<tr>
<td>COL5A2 and COL3A1</td>
<td>32</td>
<td>-1.0338</td>
<td>0.866</td>
<td>0.57</td>
</tr>
<tr>
<td>COL6A3</td>
<td>36</td>
<td>0.0472</td>
<td>0.459</td>
<td>0.36</td>
</tr>
<tr>
<td>COL9A1</td>
<td>45</td>
<td>0.1528</td>
<td>0.420</td>
<td>0.61</td>
</tr>
<tr>
<td>COL9A2</td>
<td>49</td>
<td>-0.4035</td>
<td>0.644</td>
<td>0.57</td>
</tr>
<tr>
<td>COL9A3</td>
<td>38</td>
<td>0.3390</td>
<td>0.347</td>
<td>0.36</td>
</tr>
<tr>
<td>COL10A1</td>
<td>46</td>
<td>0.1358</td>
<td>0.426</td>
<td>0.54</td>
</tr>
<tr>
<td>COL11A1</td>
<td>52</td>
<td>0.5610</td>
<td>0.273</td>
<td>0.29</td>
</tr>
<tr>
<td>COL11A2</td>
<td>47</td>
<td>0.3309</td>
<td>0.360</td>
<td>0.55</td>
</tr>
<tr>
<td>COL24A1</td>
<td>47</td>
<td>-0.0293</td>
<td>0.492</td>
<td>0.36</td>
</tr>
</tbody>
</table>

*The information content is the fraction of alleles that can be assigned unambiguously. NPL = Nonparametric linkage score from this gene was derived from multipoint NPL analysis.
lar matrix of cartilage, and it is thought that abnormal inheritance parameters, we investigated the involvement of at least 2 affected siblings, a segregation analysis to establish the mode of inheritance was not feasible. To circumvent the uncertainty about the inheritance parameters, we investigated the involvement of candidate genes by use of a linkage analysis that was model free.

Collagen is the major component of the extracellular matrix of cartilage, and it is thought that abnormal development of cartilage can result in FCP. Therefore, in the present study, collagen genes (in particular those that are involved in human bone disorders) were evaluated as candidate genes for FCP. To date, 42 collagen genes have been identified in humans. The genes COL1A1, COL1A2, COL2A1, COL3A1, COL5A1, COL6A3, COL9A1, COL9A2, COL9A3, COL10A1, COL11A1, and COL11A2 have been implicated in bone mineral density and associated with radiographically detectable osteoarthritis of the knee joint in humans could also be excluded from involvement in the development of FCP in Labrador Retrievers.

The COL1A1 gene is one of the largest collagen genes. Nonparametric analysis of the gene resulted in an NPL score of 0.36, a P value of 0.27, and allele sharing of 52%. The COL1A1 and COL2A1 genes are located 14.1 Mb apart on CFA6. Multipoint linkage analysis of the 2 markers with the FCP phenotype resulted in an NPL score of 0.18 and a P value of 0.41 for the region.

A false-negative result in the present study could have been caused by a small sample size, lack of informativity of the DNA markers, and complexity of the trait. We anticipated that the risk of obtaining a false-negative result was small because the affected dogs were Labrador Retrievers or half-breed Labrador Retrievers. Given the homogeneous genetic structure of this group of dogs and dog breeds in general, it can be inferred that the heterogeneity of complex disorders will be less and smaller numbers of affected sibling-pairs are required to detect a significant deviation from 50% allele sharing. A genome-wide scan with highly polymorphic microsatellite makers should be conducted for this disease to elucidate the genetic components. The penetrance and mode of inheritance of the gene or genes should become clear after their identification.

Olsson suggested that FCP frequently developed in breeds of dogs that had disturbances of endochondral ossification, which was speculated to be related to disturbed collagen synthesis. Our data have indicated that FCP is not caused by mutations of the collagen genes evaluated in the present study. It is possible that synthesis of 1 or more of those collagens is disturbed in an indirect manner (eg, through disturbed expression or altered posttranslational modification) in the etiology of FCP. It is also possible that the expression of collagens during development and growth is not synchronized or balanced with that of other bone-forming factors. Recently, a disturbance of bone modelling together with relative overload of the coronoid process has been suggested to be responsible for FCP. Future research on the etiopathogenesis of FCP should perhaps focus on this biomechanical aspect.

Discussion Results of the present study indicated that several Labrador Retriever and mixed Labrador Retriever-Golden Retriever litters had a high incidence of FCP, suggesting that inherited factors are involved. The affected male-to-female ratio in these litters was approximately 2:1. The sex difference of the incidence indicates that the etiology of the disorder is multifactorial. Because the litters were selected on the presence of at least 2 affected siblings, a segregation analysis to establish the mode of inheritance was not feasible. To circumvent the uncertainty about the inheritance parameters, we investigated the involvement of candidate genes by use of a linkage analysis that was model free.

Collagen is the major component of the extracellular matrix of cartilage, and it is thought that abnormal development of cartilage can result in FCP. Therefore, in the present study, collagen genes (in particular those that are involved in human bone disorders) were evaluated as candidate genes for FCP. To date, 42 collagen genes have been identified in humans. The genes COL1A1, COL1A2, COL2A1, COL3A1, COL5A1, COL6A3, COL9A1, COL9A2, COL9A3, COL10A1, COL11A1, and COL11A2 have been implicated in human skeletal or joint disorders and were included in our study. In addition, COL2A1 was investigated, and the genes COL5A2 and vitamin D receptor were included because of their proximity to COL3A1 and COL2A1, respectively. Most of the genes were analyzed through polymorphic microsatellite markers located in the vicinity. The inheritance of COL1A1, COL1A2, COL2A1, and COL11A2 was analyzed with markers at either side of the genes. Of these, the markers for COL1A1 and COL11A2 were not closely situated to the respective gene (Appendix). We calculated the genetic distances between the pairs of markers for each of the 4 genes from the Labrador Retriever family data, and in each case, the distance was approximately 10 cM (data not shown). Because recombinant dogs were excluded from the allele sharing calculation, this distance had no effect on the result.

In the present study, the DNA samples were obtained from Labrador Retrievers with radiographically and surgically confirmed FCP, dogs with other causes of elbow joint dysplasia resulting in osteoarthritis were excluded. By analyzing unaffected siblings, we could improve the information content of the sample. The sibling-pair analysis has the advantage that it does not require a model of inheritance. The analysis assesses allele sharing between pairs of affected siblings that exceeds 50%. A genetic determinant of FCP carried by a phenotypically FCP-negative dam or sire is likely to be shared by affected offspring, thereby resulting in an increase from the level of randomly expected allele sharing. The affected sibling-pair approach has been used in genetic studies of human osteoarthritis.

Results of the present study have indicated that the investigated collagen genes were not associated with high allele sharing between Labrador Retriever litters with FCP and that none of the genes is likely to play an important role in the etiology of the disorder. It should be noted that, as in the human genome, the canine gene for the vitamin D receptor is closely located to the COL2A1 gene on chromosome CFA27, with only 80 kb between the genes. Therefore, the vitamin D receptor gene (which is involved in the regulation of bone mineral density and associated with radiographically detectable osteoarthritis of the knee joint in humans) could also be excluded from involvement in the development of FCP in Labrador Retrievers.

The COL11A1 gene is one of the largest collagen genes. Nonparametric analysis of the gene resulted in an NPL score of 0.36, a P value of 0.27, and allele sharing of 52%. The COL11A1 and COL2A1 genes are located 14.1 Mb apart on CFA6. Multipoint linkage analysis of the 2 markers with the FCP phenotype resulted in an NPL score of 0.18 and a P value of 0.41 for the region.

A false-negative result in the present study could have been caused by a small sample size, lack of informativity of the DNA markers, and complexity of the trait. We anticipated that the risk of obtaining a false-negative result was small because the affected dogs were Labrador Retrievers or half-breed Labrador Retrievers. Given the homogeneous genetic structure of this group of dogs and dog breeds in general, it can be inferred that the heterogeneity of complex disorders will be less and smaller numbers of affected sibling-pairs are required to detect a significant deviation from 50% allele sharing. A genome-wide scan with highly polymorphic microsatellite makers should be conducted for this disease to elucidate the genetic components. The penetrance and mode of inheritance of the gene or genes should become clear after their identification.

Olsson suggested that FCP frequently developed in breeds of dogs that had disturbances of endochondral ossification, which was speculated to be related to disturbed collagen synthesis. Our data have indicated that FCP is not caused by mutations of the collagen genes evaluated in the present study. It is possible that synthesis of 1 or more of those collagens is disturbed in an indirect manner (eg, through disturbed expression or altered posttranslational modification) in the etiology of FCP. It is also possible that the expression of collagens during development and growth is not synchronized or balanced with that of other bone-forming factors. Recently, a disturbance of bone modelling together with relative overload of the coronoid process has been suggested to be responsible for FCP. Future research on the etiopathogenesis of FCP should perhaps focus on this biomechanical aspect.

References

b. Invitrogen, Carlsbad, Calif.
c. RHMAP, University of Michigan, Ann Arbor, Mich.
d. AmpliTaq Gold, Applied Biosystems, Foster City, Calif.
e. HEX or 6-FAM, Eurogentec, Seraing, Belgium.
f. Tandem Repeat Software, Mount Sinai School of Medicine, New York, NY.
g. Primer3 software, version 1.0, Whitehead Institute for Biomedical Research, Boston, Mass.
h. GS500 Liz or GS500 TAMRA, Applied Biosystems, Foster City, Calif.
i. GeneAnalyzer 3100, Applied Biosystems, Foster City, Calif.
j. GeneMapper software, version 3.0, Applied Biosystems, Foster City, Calif.
l. GeneHunter, Whitehead Institute for Biomedical Research, Boston, Mass.

References

Appendix

Genomic locations of candidate genes for FCP and microsatellite markers in Labrador Retrievers.

<table>
<thead>
<tr>
<th>Gene or marker</th>
<th>CFA*</th>
<th>Position†</th>
<th>Ot</th>
<th>Microsatellite PCR oligonucleotides (5'–3')</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>COL11A1</td>
<td>6</td>
<td>4,630–4,710 TSP</td>
<td>F</td>
<td>cattacaacatctcaagccgt</td>
<td>Present study</td>
</tr>
<tr>
<td>COL24A1</td>
<td>6</td>
<td>63 Mb</td>
<td>F</td>
<td>gcctatgtcctctcagt</td>
<td>Present study</td>
</tr>
<tr>
<td>KRT9</td>
<td>9</td>
<td>13.9 Mb</td>
<td>F</td>
<td>aacgccggacataggtt</td>
<td>Present study</td>
</tr>
<tr>
<td>COL1A1</td>
<td>9</td>
<td>18.6 Mb</td>
<td>F</td>
<td>aggtgctagctatctcagtt</td>
<td>Reference 39</td>
</tr>
<tr>
<td>FH2186</td>
<td>9</td>
<td>24.1 Mb</td>
<td>F</td>
<td>ttctctctctctctctctct</td>
<td>Reference 39</td>
</tr>
<tr>
<td>COL5A1</td>
<td>9</td>
<td>43.5 Mb</td>
<td>F</td>
<td>gttcctattctctctctctct</td>
<td>Reference 39</td>
</tr>
</tbody>
</table>
| COL9A1        | 12   | 36.0 Mb   | F  | gtcctttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttt