Evaluation of the potential of animal streptococcal isolates belonging to serogroups C and G to elicit acute rheumatic fever

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**Objective**—To determine whether groups C and G streptococci (GCS-GGS) isolated from animals have rheumatogenic traits associated with human GCS-GGS isolates, particularly the potential of the bacteria to interact with human collagen type IV (collagen-IV), known to be targeted during acute rheumatic fever (ARF).

**Sample Population**—64 GCS and GGS bacterial strains isolated from infected animals.

**Procedures**—Bacteria were analyzed for their ability to bind and aggregate collagen-IV and for the presence of collagen binding factors, such as the hyaluronic acid capsule, cne gene, and emm gene.

**Results**—Collagen-IV binding ability was detected in 19% (n = 12) of the isolates studied. Of the collagen-IV binding strains, 5 expressed hyaluronic acid capsule. Furthermore, emm was detected in the genome of 1 isolate, whereas all remaining collagen-IV binding isolates possessed the cne gene. Of the collagen binding factors investigated, the hyaluronic capsule was the only factor for which collagen-IV interaction could be detected. Investigation of the potential of these strains to aggregate collagen-IV revealed that animal isolates had a nonaggregating phenotype.

**Conclusions and Clinical Relevance**—Despite efficiently binding collagen-IV via hyaluronic acid, animal isolates lacked the ability to initiate aggregation of this protein. Because collagen-IV aggregation is associated with all collagen-IV–binding rheumatogenic strains, this suggested a lack of rheumatogenic potential among animal-derived GCS and GGS and, therefore, a low chance of acquiring ARF through animal contact. (Am J Vet Res 2008;69:1183–1187)

Acute rheumatic fever is an autoimmune disease that, despite its high preventability, remains one of the most serious health problems elicited by streptococci. Traditionally, GAS have been considered to be the sole causative agent of this devastating disease. However, recent evidence suggests that other species belonging to GCS and GGS are also etiologic agents for ARF. In particular, *Streptococcus dysgalactiae* subsp *equisimilis* has been implicated in a number of such cases.

The molecular mechanisms behind rheumatic fever are still not completely known, but M proteins, the major virulence factors on the streptococcal surface, are implicated in most cases. To date, data from human GCS, GGS, and some GAS rheumatogenic strains indicate that these bacteria bind and aggregate large amounts of collagen-IV either via their hyaluronic acid capsule or via certain M proteins, which are able to elicit anti–collagen-IV antibody production after immunization of mice. These M proteins are reported to share a conserved AXYLZZLN motif with high affinity toward collagen-IV, which was subsequently named PARF. Interaction of PARF with other types of collagen was also reported; however, because collagen-IV is readily soluble and easy to handle, this molecule is often preferred for collagen-binding studies.

Unlike GAS, in which single *Streptococcus pyogenes* species is restricted to the human host, GCS and GGS comprise 7 species or subspecies: *Streptococcus dysgalactiae* subsp *dysgalactiae*, *S. dysgalactiae* subsp *equisimilis*, *Streptococcus canis*, *Streptococcus equi* subsp *equi*, *Streptococcus anginosus*, and *Streptococcus phocae*, which are not only able to infect humans but also a variety of other animals. Group
C streptococci and GGS were initially reported as animal pathogens with the ability to cause zoonotic diseases, and some of the aforementioned species still remain mostly confined to particular animal species as part of their normal flora or as their main target hosts.\textsuperscript{8,10} Despite the fact that their predicted role in human disease was initially small, GCS and GGS have the ability to infect humans, with their repertoire of disease closely resembling that elicited by GAS.\textsuperscript{11} As such, GCS and GGS have been associated with diseases ranging from mild impetigo to pharyngitis to more severe invasive infections, such as streptococcal toxic shock syndrome and necrotizing fasciitis. Furthermore, GCS and GGS have been implicated in cases of streptococcal-induced autoimmune sequelae, such as glomerulonephritis and ARF.\textsuperscript{12–14}

To date, only human isolates belonging to GCS-GGS have been identified as having the potential to elicit rheumatic fever. However, most of these bacterial species can be associated with both animal and human hosts. Furthermore, episodes and even epidemics of GCS-GGS–induced glomerulonephritis correlate with previous animal contact or consumption of dairy products.\textsuperscript{18,19} The question of whether or not this rheumatogenic potential is also present among GCS-GGS animal isolates has yet to be addressed; therefore, the purpose of the study reported here was to determine whether GCS-GGS isolated from animals have rheumatogenic traits associated with human GCS-GGS isolates, particularly the potential of the bacteria to interact with human collagen-IV, known to be targeted during ARF.

**Materials and Methods**

**Bacterial strains**—Sixty-four GCS-GGS isolated from diseased farm and domestic animals were collected from veterinary facilities in the German cities of Giessen, Hannover, and Kiel from 1980 to 1987. One GAS control isolate obtained from Aachen, Germany, was used, a serotype M3 isolate (A60).

**Collagen-binding assays**—The GCS-GGS isolates were grown at 37°C overnight in trypsin soy broth. The following day, cells were washed and suspended in PBS solution to a final concentration of 10\textsuperscript{8} cells/mL. Three 250-µL aliquots of each bacterial strain were incubated for 45 minutes with 30 ng of iodine 125–labeled collagen-IV isolated from placenta.\textsuperscript{4} Collagen-IV was selected because of the high solubility and ease of handling of this molecule.\textsuperscript{3} Samples were washed in PBST, and cell-bound radioactivity was measured on a gamma counter.\textsuperscript{b} To evaluate the contribution of hyaluronic acid capsule toward collagen binding, a similar assay was performed. For each strain, sets of 6 aliquots were prepared and preincubated with either 20 µL of PBS solution or 20 µL of hyaluronidase\textsuperscript{e} for 45 minutes. Samples were washed 3 times in PBS solution prior to their incubation with radiolabeled collagen-IV.

**Emm-typing and cne detection**—The chromosomal DNA of strains with positive results for collagen-IV binding was tested for the presence of *emm* or *cne* like genes by following the protocol suggested by the CDC.\textsuperscript{20} The resulting PCR product was sequenced with the recommended primer, and a search with a basic local alignment and search tool was conducted with the CDC server.

To screen isolates for *cne*, chromosomal DNA was tested by use of PCR amplification with the primers OCNE1 and OCNE2. The reaction conditions were essentially as those described previously.\textsuperscript{21} Primer OCNE1 was used for sequencing of strain Equi1.

**CNE recombinant expression**—To facilitate the expression of CNE proteins (S containing the N-terminus region) and L (comprising the full-length mature protein), primers OCNE1, OCNE2, and OCNE3\textsuperscript{21} were redesigned to contain BamHI or SalI cutting sites. The PCR products obtained from the chromosomal DNA of strain Equi1 were digested and cloned in the pGEX-6P-1 vector,\textsuperscript{4} also previously digested with the same enzymes. The resulting expression vector was cloned into *Escherichia coli* HB101 to enable expression of recombinant proteins with an amino terminal GST-tag. Following purification of recombinant proteins with glutathione sepharose 4B\textsuperscript{b}–packed columns, the GST-tag was removed by digestion with protease.\textsuperscript{a}

**Collagen dot blot analysis**—Purified recombinant CNE proteins (S and L) as well as the M3 and M18 proteins used as positive and negative control samples, respectively, were spotted on a nitrocellulose membrane at the amount of 1, 5, and 10 µg. The membrane was blocked for 1 hour with PBST containing 5% skim milk and briefly washed in PBST before it was incubated with 30 µg of radioactive labeled collagen-IV for another hour. After this step, extensive washes with PBST were performed and a radiographic film\textsuperscript{i} was left exposed to the membrane overnight before it was developed the following day.

**Collagen aggregation**—Bacterial isolates were grown in Todd Hewitt broth supplemented with 5% yeast extract at 37°C overnight. One M3 *S pyogenes* strain was included as a positive control sample. The cells were then washed in PBS solution and resuspended to a final concentration of 10\textsuperscript{8} cells/mL. Two aliquots of 500 µL were prepared for each isolate, and while one was incubated with 40 µL of collagen-IV (1 mg/mL in 0.1M acetic acid), the other was left exposed to 40 µL of PBS solution for 30 minutes. Samples were washed in PBS solution, fixed, and processed for field emission scanning electron microscopy, as described.\textsuperscript{5}

**Results**

**Collagen-binding ability of GCS and GGS isolated from animals**—Sixty-four GCS and GGS animal isolates comprising 5 species or subspecies (*S dysgalactiae* subsp dysgalactiae, *S dysgalactiae* subsp equisimilis, *S equi* subsp equi, *S equi* subsp zooepidemicus, and *S canis*) were screened for collagen-IV binding (Table 1). Twelve (19%) isolates, all belonging to serogroup C, revealed a strong interaction with collagen-IV, and these strains were selected for further analyses. To screen for the PARF motif, collagen-IV–binding isolates were screened for *emm* genes. These isolates were also subjected to incubation with hyaluronidase to determine whether their collagen-IV–binding ability was hyaluronic acid capsule mediated (Table 2). Treatment
with hyaluronidase prevented collagen-IV binding in 5 isolates, indicating that, as reported for GCS and GGS human isolates, the hyaluronic acid capsule played an important role in the adhesion of streptococci to collagen. *Streptococcus equi* subsp *equi* and *S equi* subsp *zooepidemicus* were emm nontypable, which was not surprising because these organisms are known to have M-like proteins, which are too genetically distant from the conventional GAS M protein for traditional emm-typing methods. Sequencing of the *emm* gene from the strain Equisimilis 1 revealed a new serotype, which was not found either on the CDC server or on GenBank. This *emm* sequence type was named stC45, and it was deposited on GenBank, where it is accessible under accession No. EU159434. stC45 had very little or no homology to other *emm* types and lacked PARF, the conserved motif among collagen-IV–binding M proteins. Isolates with negative results for collagen binding were not subjected to *emm* typing because our goal in this step was to look for the presence of PARF. In case of M serotypes lacking this motif, collagen-IV–binding ability is not directly connected to the M protein; therefore, further comparisons between *emm* types of collagen-IV–binding and nonbinding isolates were not pursued. These data indicated that, although GCS and GGS animal isolates were able to bind collagen, this interaction was not fully mediated by either M protein or hyaluronic acid capsule because 7 of the collagen-binding strains in this study had neither of these structures.

### Table 1—Binding of GCS and GGS animal isolates to collagen-IV.

<table>
<thead>
<tr>
<th>Strain</th>
<th>No. of isolates</th>
<th>No. of collagen-IV-binding isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Streptococcus dysgalactiae</em> subsp <em>dysgalactiae</em></td>
<td>13 (13 GCS; 0 GGS)</td>
<td>0</td>
</tr>
<tr>
<td><em>Streptococcus dysgalactiae</em> subsp <em>equisimilis</em></td>
<td>11 (8 GCS; 3 GGS)</td>
<td>1</td>
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<tr>
<td><em>Streptococcus dysgalactiae</em> subsp <em>equi</em></td>
<td>7 (7 GCS; 0 GGS)</td>
<td>1</td>
</tr>
<tr>
<td><em>Streptococcus dysgalactiae</em> subsp <em>zooepidemicus</em></td>
<td>27 (27 GCS; 0 GGS)</td>
<td>10</td>
</tr>
<tr>
<td><em>Streptococcus dysgalactiae</em> subsp <em>canis</em></td>
<td>6 (0 GCS; 6 GGS)</td>
<td>0</td>
</tr>
</tbody>
</table>

### Table 2—Collagen-binding factors among GCS animal isolates.

<table>
<thead>
<tr>
<th>Strain</th>
<th>HA capsule</th>
<th><em>emm</em> gene</th>
<th><em>cne</em> gene</th>
</tr>
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<tbody>
<tr>
<td>Equisimilis 1</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Equi 1</td>
<td>–</td>
<td>–</td>
<td>+</td>
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<tr>
<td>Zoo 1</td>
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<tr>
<td>Zoo 10</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
</tbody>
</table>

HA = Hyaluronic acid. + = Present. – = Absent. Zoo = Zooepidemicus.

### Figure 1—Dot-blot analysis for CNE protein in *Streptococcus equi*.

Recombinant CNE-S and CNE-L were spotted (1, 5, and 10 µg) and tested for binding to radiolabeled collagen-IV. M3 and M18 were used as reference positive and negative control samples.

### Figure 2—Field emission scanning electron micrographic views of an M3 *Streptococcus pyogenes* (A) and *Streptococcus dysgalactiae subsp equisimilis* (B), which had previously been incubated with collagen-IV. Aggregation is only visible in the M3 positive control sample. Bar = 1 µm.

CNE and collagen IV binding—In *S equi* subsp *equi* and *S equi* subsp *zooepidemicus*, a protein named CNE has been reported to bind collagen type I. To determine whether CNE was the factor conferring collagen-IV–binding ability to M protein and hyaluronic acid negative collagen-binding strains, the presence of
cne was investigated via PCR assay. The gene was found in all collagen-IV–binding isolates belonging to S equi subsp equi and S equi subsp zooepidemicus, irrespective of the presence or absence of hyaluronic acid capsule (Table 2). For all samples, the amplified product was of the expected size. To confirm the identity of the gene, 1 sample (Equi 1) was selected for DNA sequencing analysis (data not shown). Recombinant proteins representing both the N-terminus and the full-length CNE protein from the sequenced Equi 1 isolate were found to interact with collagen-IV by use of dot-blot analysis (Figure 1), although to a lesser extent than the M3 positive control sample.

GCS animal isolates and collagen-IV aggregation—So far, all collagen-IV–binding rheumatogenic strains have been reported to not only bind collagen-IV, but also to aggregate it at their cell surface. Therefore, to determine whether collagen-binding isolates in this study have rheumatogenic potential, bacteria were analyzed for collagen-IV aggregation. Despite their efficient collagen-binding ability, comparable to the levels obtained for human rheumatogenic strains, none of the isolates were able to aggregate a detectable amount of collagen-IV when observed via electron microscopy. The phenotype obtained with an M3 S pyogenes strain was compared with that obtained with GCS and GGS isolates (Figure 2). Of particular note was the fact that the capsule expressed by GCS animal isolates seemed to differ from the capsule expressed by M18 GAS strains, which had been reported to aggregate collagen-IV. Thus, it appears that the collagen-IV–binding ability of the animal GCS isolates in this study, regardless of the mechanisms via which it is mediated, did not correlate with a rheumatogenic potential of these strains.

Discussion

A number of ARF episodes have been attributed to S dysgalactiae subsp equisimilis infections, and it has been suggested that these strains, isolated from humans, might induce this disease by aggregating collagen-IV and eliciting the production of anti–collagen-IV antibodies. In the present study, the potential of these and other animal-derived species of groups C and G streptococci to do the same was investigated. Results indicated that these isolates were also capable of collagen-IV binding, although to a lesser extent (19%) than for GCS and GGS isolates associated with human infection (38%). It should be noted that bacteria in the previous study were recovered from a broader geographic area that included both endemic and nonendemic areas for rheumatic fever. However, even among isolates from nonendemic areas, collagen-IV binding as well as rheumatogenic potential was encountered.

Regarding known collagen-IV–binding factors, the hyaluronic acid capsule was the only factor shared by human and animal GCS and GGS strains. An emm gene was also detected in one of the isolates, but binding ability was completely abolished upon incubation with hyaluronidase, indicating that the capsule and not the M protein was the factor mediating the collagen-IV interaction. Moreover, the newly found stC45 serotype did not contain the AXYLZZLN PARF motif found in collagen-IV–binding rheumatogenic M proteins. The CNE protein seems to be a strong candidate as a putative collagen-IV interacting partner. However, further studies on this protein need to be conducted to determine whether the high levels of collagen binding seen in several strains in this study were mediated by CNE. Screening of S equi subsp equi and S equi subsp zooepidemicus isolates that did not bind collagen also revealed a cne gene (data not shown). This may indicate that collagen-binding factors other than CNE mediate collagen binding by the isolates studied here or that regulation and expression of cne, rather than the gene itself, are important indicators of collagen-binding potential. Investigation of the collagen-IV–binding ability of recombinant CNE indicated that this protein had a lower binding ability than the recombinant M3 protein, suggesting that the lack of collagen-IV aggregation by GCS and GGS seen in this study was attributable to the lower affinity of CNE for collagen-IV, rather than downregulation of cne expression. This hypothesis is currently being investigated; however, the constitutive expression of unspecific IgG-binding protein G by all of these isolates and reports of CNE inactivation upon release from the cell surface provide substantial challenges for this line of study.

Irrespective of the mechanisms via which collagen binding is mediated, all of the collagen-binding isolates identified in this study were unable to aggregate collagen. This suggests that these strains lacked the ability to elicit ARF via this mechanism. Whether or not these strains have the potential to induce ARF via other mechanisms, such as mimicking a human protein in an interaction similar to that of S pyogenes proteins and human myosin, remains to be investigated.

The fact that the hyaluronic acid capsule expressed by the GCS-GGS animal isolates did not enable detectable collagen-IV aggregation could be related to a lower level of expression of hyaluronic acid on these bacteria. It is known that M18 strains are highly encapsulated, so it is possible that lower concentrations of hyaluronic acid are sufficient for collagen-IV binding, but higher levels are required for an aggregation phenotype. It is not known whether animal strains are able to upregulate their capsule expression after adaptation to the human in vivo environment.

There has been an increase in cases of disease caused by GCS and GGS infections, but whether this increase is related to an increase in virulence of GCS and GGS toward humans, an improvement in diagnostic methods to identify these species, or both is not clear. Horizontal gene transfer is a mechanism that is known to occur between GAS and GCS-GGS, and may explain a potential increase in pathogenicity of GCS and GGS. Given the reported confinement of GAS to the human host, it is likely that gene transfer would occur during the coinfection of humans by GAS and GCS-GGS. The occurrence of horizontal gene transfer may explain the finding that, in comparison to a previous study of human GCs and GGS isolates, S equisimilis human isolates have much greater potential to bind collagen-IV (38%) than do S dysgalactiae subsp equisimilis animal isolates (9%).

Conversely, certain GCs and GGS species, such as S dysgalactiae subsp dysgalactiae or S equi subsp equi,
seem to be more restricted to selected animal species,\(^8\) and the likelihood of them encountering a GAS strain for gene transfer to occur is therefore reduced. The existence of these distinct ecologic niches may therefore be the reason these particular GCS and GGS species have a lower ability to elicit human disease, compared with their counterpart \(S\). dysgalactiae subsp equisimilis.\(^6\)

So far, GCS- and GGS-related cases of ARF have strictly been reported to be caused by \(S\). dysgalactiae subsp equisimilis, and from our findings, it seems reasonable to speculate that, among this species, only the human isolates have the ability to elicit this disease. This suggests that \(S\). dysgalactiae subsp equisimilis isolates acquire a rheumatogenic potential only upon colonization of a human host and that either this potential is lost once the bacteria is transmitted back to a different animal species or, simply, that the likelihood of a human-to-animal transmission is so reduced that in our study, we were not able to detect any animal isolate with this potential. In this respect, it might be beneficial to conduct a similar study of animal isolates from a variety of geographic locations, where ARF rates are higher and contact between humans and animals is more frequent. Furthermore, because it is also known that most streptococcal gene transfers appear to be mediated by phages\(^{21}\) and that, at least for GAS to GAS transfers, the role of a secreted mammalian host protein in inducing phage production seems to be crucial,\(^{26}\) it would also be of great interest to know if this phage transfer mechanism is the same for GAS to GCS-GGS and GCS-GGS to GCS-GGS transfers or if the factor that induces phage production exercises its activity solely upon GAS. This latter scenario, compatible with a known preferential unidirectional transfer of genes from GAS to GCS-GGS,\(^{24}\) would also restrict gene spread among animal isolates, making the traits associated with the human isolates difficult to detect among the animal isolates. Although one should be cautious of excluding the possibility that GCS and GGS associated with animal infections have the potential to elicit ARF, data reported here suggest that the ability of these isolates to initiate this disease is more restricted than the ability of human isolates of these bacteria.

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