**Characterization of Streptococcus pyogenes from Animal Clinical Specimens, Spain**

Ana Isabel Vela, Pilar Villalón, Juan Antonio Sáez-Nieto, Gema Chacón, Lucas Domínguez, José Francisco Fernández-Garayzábal

_S. pyogenes_ appears to be almost exclusively restricted to humans, with few reports on isolation from animals. We provide a detailed characterization (emm typing, pulsed-field gel electrophoresis [PFGE], and multilocus sequence typing [MLST]) of 15 _S. pyogenes_ isolates from animals associated with different clinical backgrounds. We also investigated erythromycin resistance mechanisms and phenotypes and virulence genes. We observed 2 _emm_ types: _emm_12 (11 isolates) and _emm_77 (4 isolates). Similarly, we observed 2 genetic linages, sequence type (ST) 26 and ST63. Most isolates exhibited the _M_ macrolide resistance phenotype and the _mefA_ and _ermB_ genotype. Isolates were grouped into 2 clones on the basis of _emm_-MLST-PFGE-virulence gene profile combinations: clone 1, characterized by the combined genotype _emm_12-ST36-pulsotype A-speG; and clone 2, characterized by the genotype _emm_77-ST63-pulsotype B-speC. Our results do not show conclusively that animals may represent a new reservoir of _S. pyogenes_ but indicate the ability of human-derived _S. pyogenes_ isolates to colonize and infect animals.

**Materials and Methods**

**Origin and Identification of Bacterial Isolates**

We analyzed 15 isolates of _S. pyogenes_ obtained from rabbits (n = 14) and sheep (n = 1) in Spain during 2006–2014 (Table 1). Most rabbit isolates were from unrelated animals, located in different commercial farms (n = 14) and locations throughout Spain. Links between rabbit farms were not identified. The sheep included in this study was from a farm that had no rabbits. Human contact with animals was restricted to the personnel working in the rabbit farms and sheep flocks.

We recovered isolates from different clinical backgrounds: 8 from skin infections, 4 from genital tract infections, and 1 each from respiratory infections, mastitis, and otitis. We collected samples from skin and ear infections with sterile cotton swabs and collected the milk sample from the mastitis case aseptically in a sterile tube. Rabbits with genital tract or lung infections were euthanized, at farms or laboratories, and necropsied under aseptic conditions; clinical specimens were collected with forceps and scissors scrubbed in 70% ethanol. Samples taken at farms were transported to the laboratory in refrigerated polyethylene bags and processed within 24 hours after sampling.

Clinical specimens were sampled onto blood agar plates that were incubated at 37°C for 24–48 hours. Identification of isolates as _S. pyogenes_ was based on colony morphology, β-hemolysis, and biochemical characteristics using the commercial identification system rapid
Table 1. Features and disease manifestations of 15 animals from which Streptococcus pyogenes isolates were collected, Spain, 2006–2014

<table>
<thead>
<tr>
<th>Isolate*</th>
<th>Animal</th>
<th>Clinical background</th>
<th>Specimen</th>
<th>Geographic region</th>
<th>Isolation date†</th>
</tr>
</thead>
<tbody>
<tr>
<td>M50163</td>
<td>Rabbit</td>
<td>Metritis</td>
<td>Uterus</td>
<td>Valencia</td>
<td>2006 Jan</td>
</tr>
<tr>
<td>M79144</td>
<td>Rabbit</td>
<td>Abscesses and dermatitis</td>
<td>Skin</td>
<td>Valladolid</td>
<td>2013 Mar</td>
</tr>
<tr>
<td>M78761</td>
<td>Rabbit</td>
<td>Dermatitis</td>
<td>Skin</td>
<td>Valladolid</td>
<td>2013 Feb</td>
</tr>
<tr>
<td>M75791</td>
<td>Rabbit</td>
<td>Abscesses</td>
<td>Skin</td>
<td>Valencia</td>
<td>2012 Apr</td>
</tr>
<tr>
<td>M75539</td>
<td>Sheep</td>
<td>Abscesses</td>
<td>Skin</td>
<td>Zaragoza</td>
<td>2012 Mar</td>
</tr>
<tr>
<td>M75533</td>
<td>Rabbit</td>
<td>Otitis</td>
<td>Ear</td>
<td>Valencia</td>
<td>2012 Mar</td>
</tr>
<tr>
<td>M75123</td>
<td>Rabbit</td>
<td>Metritis</td>
<td>Uterus</td>
<td>Castellón</td>
<td>2012 Feb</td>
</tr>
<tr>
<td>M73512</td>
<td>Rabbit</td>
<td>Abortion</td>
<td>Uterus</td>
<td>Zaragoza</td>
<td>2011 Aug</td>
</tr>
<tr>
<td>M72636</td>
<td>Rabbit</td>
<td>Metritis</td>
<td>Uterus</td>
<td>Zaragoza</td>
<td>2011 May</td>
</tr>
<tr>
<td>M72193</td>
<td>Rabbit</td>
<td>Abscesses</td>
<td>Skin</td>
<td>Valencia</td>
<td>2011 Apr</td>
</tr>
<tr>
<td>83639</td>
<td>Rabbit</td>
<td>Abscesses and dermatitis</td>
<td>Skin</td>
<td>Valladolid</td>
<td>2014 Mar</td>
</tr>
<tr>
<td>83553</td>
<td>Rabbit</td>
<td>Pneumonia</td>
<td>Lung</td>
<td>Zaragoza</td>
<td>2014 Mar</td>
</tr>
<tr>
<td>M82209</td>
<td>Rabbit</td>
<td>Abscesses</td>
<td>Skin</td>
<td>Valladolid</td>
<td>2013 Dec</td>
</tr>
<tr>
<td>M75768</td>
<td>Rabbit</td>
<td>Mastitis</td>
<td>Milk</td>
<td>Zaragoza</td>
<td>2012 Mar</td>
</tr>
<tr>
<td>85374</td>
<td>Rabbit</td>
<td>Skin infection</td>
<td>Skin</td>
<td>Valladolid</td>
<td>2014 Aug</td>
</tr>
</tbody>
</table>

*Isolates M50163 and M73512 were recovered in pure culture. The remaining isolates were recovered together with Staphylococcus aureus.
†Except for isolates M79144 and M78761, which were isolated in the same farm but at different times, all other isolates were recovered from animals at different farms.

ID 32 STREP (BioMerieux, Marcy L’Étoile, France). Biochemical identification was also confirmed by sequencing the 16S rRNA gene (8).

**Antimicrobial Drug Susceptibility Tests**

We performed drug susceptibility testing using the Clinical and Laboratory Standards Institute broth microdilution method (9) in Mueller–Hinton broth supplemented with 5% lysed horse blood. We determined the susceptibilities of the isolates with a commercially available susceptibility test (CMV3AGPF Sensititer standard panel; Trek Diagnostics, West Essex, UK) performed according to the manufacturer’s instructions. The agents we tested were penicillin (0.25–16 μg/mL), erythromycin (0.25–8 μg/mL), vancomycin (0.25–32 μg/mL), daptomycin (0.25–16 μg/mL), chloramphenicol (2–32 μg/mL), linezolid (0.5–8 μg/mL), tetracycline (1–32 μg/mL), quinupristin (0.5–32 μg/mL), tigecycline (0.05–0.5 μg/mL), streptomycin (512–2048 μg/mL), kanamycin (128–1024 μg/mL), lincomycin (1–8 μg/mL), and gentamicin (128–1024 μg/mL). In addition, we determined MICs of clindamycin, erythromycin, and tetracycline by Etest (AB Biodisk, Solna, Sweden). We interpreted the results using the Clinical and Laboratory Standards Institute breakpoints for streptococci (9) for penicillin, erythromycin, vancomycin, daptomycin, chloramphenicol, tetracycline, and quinupristin; the European Committee on Antimicrobial Susceptibility Testing breakpoints for tigecycline and linezolid (http://www.eucast.org/clinical_breakpoints); and the Comité de l’Antibiogramme de la Société Française de Microbiologie breakpoints (10) for streptomycin, kanamycin, lincomycin, and gentamicin.

**Macrolide Resistance Phenotype**

To identify macrolide resistance phenotypes, we used a double-disk diffusion test (D-zone test) using erythromycin (15 μg) and clindamycin (2 μg) disks, as described by Hasenbein et al. (11). Isolates with blunting of the clindamycin inhibition zone around the disk adjacent to the erythromycin disk were considered to have an iMLSb phenotype (erythromycin resistant and clindamycin inducible). Clindamycin-susceptible isolates without blunting indicated an M phenotype (erythromycin resistant and clindamycin susceptible). Isolates that were resistant to both antimicrobial drugs were considered to have a cMLSb phenotype (constitutive erythromycin and clindamycin resistant).

**Detection of Macrolides and Tetracycline Resistance Genes**

We extracted DNA according to the protocol in the US Centers for Disease Control and Prevention (CDC) S. pyogenes sequence database (http://www.cdc.gov/ncidod/biotech/strep/protocols.htm). We screened all erythromycin-resistant isolates by PCR for the erythromycin resistance genes emrB (12), emrA (13), mefA (14), and msrD (15). We tested tetracycline-resistant isolates for the tetracycline resistance genes tetM and tetO (14).

**Detection of Virulence Genes**

We tested the S. pyogenes isolates for the presence of the virulence genes speA, speB, speC, speF, speG, speH, speI, speM, ssa, and smeZ by PCR. We used primers and conditions described previously (16,17).

**PFGE Analysis, MLST, and emm Typing**

For PFGE analysis, genomic DNAs of the S. pyogenes isolates were prepared and digested with Smal restriction enzyme (MBI Fermentas, Vilnius, Lithuania) following a previously published protocol (18). We performed MLST following the method established by Enright et al. (19) and assigned the allele and sequence type (ST) according to the PubMLST website (http://pubmlst.org/spyogenes). We amplified and sequenced the emm gene according to the
Results
We observed 2 emm types (Table 2): emm12 was the most frequent (11 isolates), followed by emm77 (4 isolates). Two pulsortypes (A and B) were generated after typing the isolates by PFGE with the restriction enzyme SmaI; 11 isolates were pulsortype A and 4 isolates pulsortype B (Figure). Similarly, we observed 2 genetic lineages (ST26 and ST63) after MLST analysis.

All 15 S. pyogenes isolates were susceptible to penicillin (MIC ≤0.25 mg/L), vancomycin (MICs ≤0.25 to 0.5 mg/L), daptomycin (MIC ≤0.25 mg/L), chloramphenicol (MICs ≤2 to 4 mg/L), tigecycline (MICs ≤0.015 to 0.12 mg/L), and gentamicin (MIC ≤128 mg/L). Additionally, all isolates but 1 were susceptible to kanamycin (MIC ≤128 mg/L), and 12 isolates showed susceptibility to linezolid (MICs <2 mg/L), streptomycin (MICs ≥2.048 mg/L), and lincomycin (≥8 mg/L). On the other hand, all isolates were resistant to tetracycline, with MICs ranging from 24 to 96 mg/L using Etest (Table 2). Eleven isolates showed tetracycline-resistant genotypes tetM/tetO, 2 isolates tetO, and 1 isolate tetM (Table 2).

Most isolates (7/15) exhibited the M phenotype, 2 isolates the phenotype cMLSγ, and 1 the phenotype iMLSγ (Table 2). The macrolide-resistant genotype mefA/ermB was the most frequently observed, seen in all isolates but 1 with the M phenotype and in the isolate with phenotype cMLSγ. The genotype ermB was observed alone in 1 isolate of each phenotype. No isolate carried the msrD or ermA macrolide-resistant determinants.

We detected the chromosomal-encoded speB and speF genes in all isolates. We observed 2 different virulence gene profiles based on the presence/absence of the speG and speC genes. We detected the genotype speG in 11 isolates and the genotype speC in 4 isolates (Table 2).

We grouped the 15 S. pyogenes isolates into 2 different clones on the basis of emm-MLST-PFGE-virulence genes profile combinations. Clone 1 grouped isolates characterized by the combined genotype emm12-ST36-pulsortype A-speB/speF/speG, whereas isolates of clone 2 were characterized by the genotype emm77-ST63-pulsortype B-speB/speF/speC (Table 2). In addition, isolates of clone 1 were erythromycin resistant, mainly exhibiting an M phenotype, and isolates of clone 2 were erythromycin susceptible.

Discussion
S. pyogenes is a human pathogen that has rarely been isolated from animals. It has been isolated from abscesses in cervical and mesenteric lymph nodes and liver of a free-living European hedgehog (E. europaeus) and from 2 dogs with severe colonic disease and conjunctivitis (5–7). Here we describe the detailed characterization of animal S. pyogenes isolates from different clinical specimens obtained from rabbits (n = 14) and sheep (n = 1) in Spain during 2006–2014. This pathogen was recovered mainly from noninvasive cases, with skin infections being the most common clinical presentation (n = 6), followed by genital tract infections (n = 4) (Table 1). S. pyogenes was isolated from all skin clinical samples together with Staphylococcus aureus, a well-recognized pathogen associated with different skin diseases in animals (20). These results indicate that although S. pyogenes should be able to colonize the skin of animals, it is difficult to ascertain its etiologic significance in skin infections. However, S. pyogenes was isolated in pure culture from clinical specimens of the genital tract, ears, mammary glands, and lungs in rabbits, indicating the potential role of S. pyogenes in these infections.

Most of the S. pyogenes isolates we tested (n = 11) exhibited the genotype emm12-ST36, which has been isolated...
repeatedly from humans in different countries (21–27), including Spain (28–30). This genotype can exhibit an M phenotype (31) and has been associated with skin and soft tissue infections (32), data that fit with our results, as more than half of the isolates with this genotype were isolated from abscesses and dermatitis (Table 2). The genotype emm77-ST63 that we identified in 4 animal isolates has also been detected in human S. pyogenes isolates (21,25,33), but unlike human isolates, the isolates in our study were erythromycin and clindamycin susceptible (Table 2).

All 11 isolates in clone 1 (pulsotype A) exhibited PFGE profiles that were indistinguishable from each other, and all 4 isolates in clone 2 also exhibited PFGE profiles that were indistinguishable PGFE from each other (pulsotype B; Figure). Isolates of S. pyogenes usually exhibit high levels of genetic diversity (4). Thus, the fact that we identified only 2 clones in different isolates collected over a period of 8 years was unexpected. The possibility of a common source of infection is very unlikely because all isolates were recovered at different times from different animals in farms located at geographically distant locations spread throughout Spain, without any epidemiologic relationship (Table 1). In addition, clinical specimens were processed independently in the same laboratory by highly qualified and trained personnel, which makes the possibility of a cross-contamination in the laboratory unlikely.

Under these conditions, multiple human-to-animal transmission events should be the most likely origin of these genotypes in sheep and rabbits. Another possible explanation could be that genotypes ST36 and ST63, although originating from humans, represent genetic linages that were indistinguishable PGFE from each other (pulsotype B; Figure). Isolates of S. pyogenes emm77-ST63 that we identified in 4 animal isolates has also been detected in human S. pyogenes isolates (21,25,33), but unlike human isolates, the isolates in our study were erythromycin and clindamycin susceptible (Table 2).

Isolates with the genotype mefA/ermB usually correlate with the cMLSb phenotype, but 5 of the 6 S. pyogenes isolates with the mefA/ermB genotype in our study exhibited M phenotype (Table 2), which agrees with previous observations (29). The erm gene usually confers co-resistance to macrolides, lincosamides, and streptogramins. Curiously, all M phenotype isolates in our study showed susceptibility to clindamycin and were positive for the ermB gene. This result, although unusual, has also been observed previously in S. pyogenes isolates from different countries (26,36–38). A possible explanation could be that the ermB gene was nonfunctional in the isolates with clindamycin-susceptible phenotypes. The isolate M72193 exhibited the iMLSb phenotype but was ermA-negative (Table 2). This result, although infrequent, has also been observed in previous studies (39). Isolates with the iMLSb phenotype have been further subdivided into 3 distinct types: type A, associated with the presence of the ermB gene; and types B and C, associated with the presence of the ermA gene (40,41). This isolate carried the ermB gene (Table 2), suggesting therefore an iMLSb-A phenotype.

Unlike most human S. pyogenes isolates, which usually carry either tetM or tetO genes, most of the isolates in this study (n = 11) carried both genes (Table 2). Human isolates with the combination of tetM and tetO tetracycline-resistance genes have been identified previously in Spain (29). Another uncommon result was the identification of 1 isolate (83553) that was resistant to tetracycline (MIC 64 mg/L) but lacked resistance tetM and tetO genes (Table 2) commonly associated with tetracycline resistance in S. pyogenes (42). However, tetracycline-resistant strains and negativity to these genes have also been reported (43). Further studies will be necessary to elucidate the precise mechanism of resistance to tetracycline in this strain.

In summary, this study provides a detailed characterization of animal S. pyogenes isolates associated with
different clinical backgrounds. This pathogen should be considered by veterinary microbiologists when processing clinical material from animals.

Dr. Vela is an associate professor at the Animal Health Department, Veterinary Faculty, Complutense University, Madrid, Spain. Her research focuses on the characterization of relevant animal bacterial pathogens.

References