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TRANSLATIONAL RESEARCH

Genomic Study on Blood Culture Isolates From Patients With *Staphylococcus* Infection-associated Glomerulonephritis

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Introduction: *Staphylococcus* infection-associated glomerulonephritis (SAGN), is an autoimmune sequela of infection affecting a subset of infected patients without specific predictive factors, frequently presenting with acute nephritic syndrome and propensity for chronic kidney disease. We performed a comparative genotypic and phenotypic analysis of *S. aureus* isolates from patients that did and those that did not develop SAGN.

Methods: We had 22 culture-proven cases of SAGN from Ohio State University Wexner Medical Center (OSUWMC) from 2004 to 2016, 9 of 22 being blood cultures, with archived isolates. These, along with blood culture isolates from 12 patients with no clinical evidence of SAGN (between ages 40 to 80 years) over the same period were used for genotyping. For host demographic comparison, we used all available SAGN cases ($n = 85$, including those with positive cultures other than blood; and patients with kidney biopsies received from referring hospitals) and all OSUWMC patients with positive *Staphylococcus* cultures without glomerulonephritis (GN) ($n = 23,496$).

Results: Multiple sequence types (STs) suggesting strain diversity was seen in the GN isolates with mainly clonal complexes (CC) 5 and 59. Mutations in the *agr* operon were identified in significantly higher number of the GN isolates (83%) than non-GN isolates (16%). Significant differences in β-hemolysis and biofilm formation was also observed between the groups.

Conclusion: The functionality of these *agr* mutants remains to be seen, but the presently known effects of reduced *agr* function, namely increased surface adhesins, biofilm formation, and persistent bacteremia could be important microbial factors predisposing to SAGN and testing for them early during infection could help to predict its development.

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Since the 1960s, methicillin-resistant *Staphylococcus aureus* (MRSA) has emerged globally as a leading cause of healthcare and community-associated infections.[1-4] In a subset of patients with infection, autoimmune sequelae can develop, particularly SAGN or toxic-shock syndrome.[5-11] Our study focuses on characterizing microbial genotypes and host demographic characteristics in SAGN.

SAGN usually affects the middle age to elderly with associated comorbidities, such as diabetes mellitus, artificial heart valves, invasive central lines, other prosthetic and orthopedic devices, post-surgery or multiple trauma, or i.v. drug abuse.[6-9] Interestingly, the infection is usually extrarenal. Patients often require prolonged supportive care for acute kidney injury, and extended course of antibiotics (and in some cases surgical treatment for endocarditis or heart valvular abscess and infected limbs in patients with diabetes). Avoidance of immunosuppressive drugs is important despite presence of active GN, making SAGN a unique form of GN. The time period between onset of infection and onset or presentation of GN can be variable but without a definite infection-free latent period in between.[5-11] Interestingly, only a subset of patients with infection develop this complication, even among the elderly patients with comorbidities. Although, *Staphylococcal* enterotoxins and superantigens are thought to play a role in pathogenesis,[5] there are no known predictive markers, particularly microbial factors.

This is a retrospective exploratory study done in 2 parts. The first part involves comparing host demographic features and selected laboratory parameters between patients with SAGN and *S. aureus* infected patients without SAGN. The second part involves genotyping and phenotyping of archived *S. aureus* blood culture isolates of patients that did and those that did not develop SAGN, to look for potential differences, using whole genome sequencing (WGS) and *in vitro* functional assays. The bacteriologic study was limited to archived isolates from blood culture specimens from our institution alone. Host demographic study was done on a much larger patient cohort, including those who had positive cultures other than blood and patients from multiple referring hospitals whose kidney biopsies were sent to OSUWMC for diagnosis.

METHODS

Patient Demographics

Over a 13-year period (2004–2016), we identified a total of 85 cases from our biopsy database (Figure 1), with a definitive diagnosis of SAGN,[7,8] according to diagnostic criteria described before.[9] These included patients managed in our medical center, OSUWMC, and patients whose biopsies were received at OSUWMC Renal Pathology Division from multiple referring hospitals across the United States for diagnostic workup. As a comparison group, we identified patients from OSUWMC between 2008 to 2011, with positive *Staphylococcus* cultures, but no clinical evidence of SAGN, using OSUWMC Information Warehouse. We identified 23,496 unique patients with a total of 27,777 positive *Staphylococcus* culture results (mainly *S. aureus* and few *S. epidermidis*, including blood and/or other specimen types). Demographic parameters (age and sex) were statistically compared for this entire cohort of GN (n = 85) and non-GN (n = 23,496) patients. Spectrum of infections and host comorbidities have been extensively described in previous studies by our group and others, therefore not elaborated here.[5-9]

This study was approved by The Ohio State University Internal Review Board (IRB 2017H0081; IRB 2020H0557); (MTA 2021-1330; MTA 2017-2292).

Laboratory Parameters

Routinely tested laboratory parameters were retrospectively assessed to determine if these showed any significant differences between the GN and non-GN infected patients. These included serum creatinine, and total and differential white blood cell counts from 2 different time points, at the time of first microbiological culture and then 2 weeks following initial culture (ranged from 10 to 20 days). The 2-week cutoff was arbitrary, but it helped to capture the trends during the hospital stay. The parameters were also recorded on the day of the biopsy along with the timing of biopsy (Table 1). This data was available only on patients admitted and managed at OSUWMC (22 of 85; as indicated in Figure 1), and were retrieved by retrospective electronic chart review. For the comparison group, we retrieved similar data for 96 patients from the non-GN cohort, for whom laboratory data at the required time points was available (detailed data not shown, only statistical comparisons shown). To minimize bias, we selected patients between 40 and 70 years age group, including males and females, similar to the age prevalence among patients with SAGN (Supplementary Table S1). Low serum C3 is known to occur in a subset of patients with SAGN,[6-9] but these were not routinely tested in patients without GN, therefore not studied here.

Bacteriologic Study

As shown in Figure 1, 9 of the 22 OSUWMC patients with SAGN had positive blood cultures and per OSUMC protocol, their isolates had been preserved as glycerol stocks and were available for this study (SAGT-13–SAGT-21; Table 2). Wound, skin, or respiratory cultures are not routinely archived and were unavailable for the study. *S. aureus* blood culture isolates of 12 patients from the non-GN patient cohort were used as comparator group (SAGT-1–SAGT-12; Table 2). Criteria for the comparator group included absence of clinical features of GN (hematuria and proteinuria), similar spectrum of infections, patient age mainly in the 40 to 70 years group, and date of culture

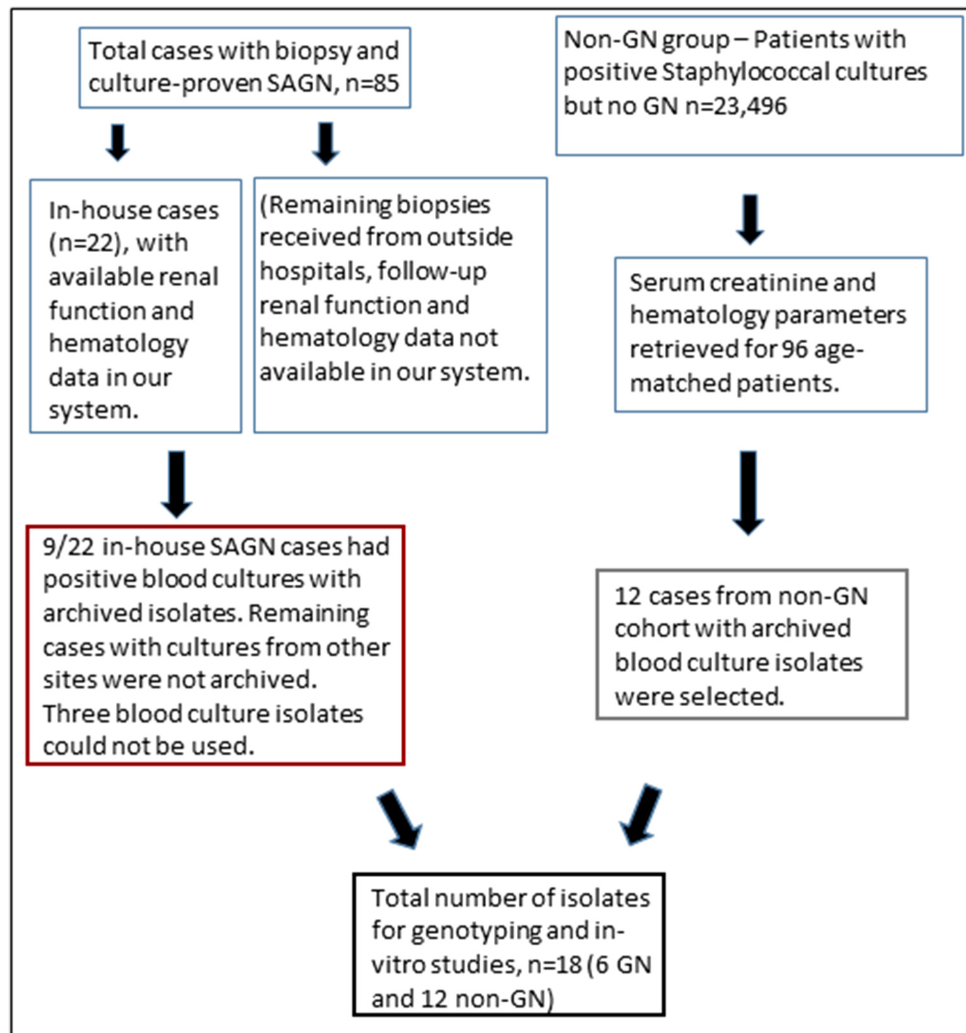


Figure 1. Flowchart showing total number of patients with and without GN and those available for genotyping studies. GN, glomerulonephritis; SAGN, *Staphylococcus* infection-associated glomerulonephritis.

during the same years as the 9 GN isolates (2014–2016). The archived isolates were plated on blood agar and incubated for 24 hours. A second subculture was performed on day 2 on tube agar. After allowing growth for 24 hours, they were sent for genotyping studies, and a similar batch for *in vitro* functional assays. Two of the archived isolates (SAGT-15 and SAGT-19) unfortunately were not available for testing (disposed after a certain time period as per regulations of the Clinical Microbiology laboratory). One isolate (SAGT-16) developed contamination and was excluded, leaving 6 of 9 SAGN isolates for the genotypic and phenotypic analysis.

Whole Genome Sequencing

WGS was performed at the Ohio Department of Agriculture which maintains an ISO 17025-accredited WGS program for bacteria isolates. GalaxyTrakr, a distributed analysis tool for WGS data was utilized to assess the quality, taxonomy, and possible contamination of each sample. The GalaxyTrakr MicroRunQC workflow was used to check WGS sequence quality, following the protocol available within the US Food and Drug Administration GenomeTrakr protocols.io workspace (https://www.protocols.io/view/assessing-sequence-quality-in-galaxytrakr-bdvfi63n)[12-15] (Supplementary Methods).

WGS Data Analysis

A total of 18 paired end sequences were available for the WGS study (SAGN $n = 6$; non-SAGN $n = 12$; Table 3). Prealignment quality control with Fastqc (quality control tool for high throughput sequencing data) showed no quality issues and no adapter content. We used Pathosystems Resource Integration Center (PATRIC) to perform comprehensive genome analysis, including assembly, annotation, and phylogenetic tree generation. Assembly was performed using unicycler and annotation with rapid annotation using subsystem technology (Supplemental Methods).

Table 1. Total and differential WBC counts and serum creatinine levels of in-house patients with SAGN (N = 22)

	On day of culture				2 weeks post culture date				On the day of the kidney biopsy				
	Total WBC count/ul	PMNs %	Lymphocytes %	Serum Creatinine mg/dl	Total WBC count/ul	PMNs %	Lymphocytes %	Serum Creatinine mg/dl	Timing of biopsy in relation to 2 wk interval	Total WBC count/ul	PMNs %	Lymphocytes %	Serum Creatinine mg/dl
1	10,000	73.5	2.6	0.9	3500	86.8	6.6	7.3	after	3400	60.9	20.6	2.1
2	19700	80.3	4.7	1.0	10800	65.1	21.6	1.5	after	6100	54.3	30.5	6.1
3	8100	74.9	12.3	1.2	7400	75.5	13.6	1.0	after	9600	75.7	12.2	3.4
4	8500	73.2	14.5	0.8	6900	82.8	8.9	0.6	after	15100	91.6	3.2	1.9
5	19000	87.5	4.4	1.3	9500	62.7	24.9	1.4	after	11400	80.3	8.4	2.0
6	11600	70.7	13.9	1.1	8000	74	17.9	2.1	after	6400	54.6	29.6	3.6
7	16000	89.1	5.6	1.2	24000	80.1	9.7	2.3	after	12800	81.1	6.6	3.2
8	8200	76	14	1.2	7200	75.8	13.7	1.1	after	9900	82.1	8.1	4.3
9	41400	81	0.7	0.9	9500	77.3	6.0	2.8	after	7700	77.3	6.0	2.6
10	8300	68.1	15.5	1.0	6000	63.4	18.9	1.3	after	6100	60.6	19.6	4.8
11	17,300	90.7	2.0	3.2	9700	69.6	16.0	4.1	between	10,000	83.9	7.7	4.0
12	8700	73.3	17.6	1.7	9400	67.9	22.1	1.9	between	8800	61.7	20.4	2.0
13	5400	53.9	28.1	3.6	3600	54.2	25.0	3.9	between	3700	40.2	31.9	3.6
14	15,400	84.1	8.0	0.6	32700	88.7	0.7	0.5	between	30,400	93.2	2.0	1.0
15	17,900	77.7	13.0	0.9	7900	67.6	18.5	1.9	between	9600	77.4	12.7	2.2
16	5200	49	26.0	3.8	8700	64	18.6	5.8	between	7000	61.3	23.7	5.6
17	10800	76.9	12.2	9.5	8000	73.6	13.3	6.5	between	10300	76.2	12.2	6.0
18	8900	92	3.4	3.7	8500	77.2	11.8	3.9	between	10800	78.2	12.3	6.7
19	4200	51.5	31.4	4.6	5700	62.9	21.4	6.8	between	3900	62.9	21.4	6.9
20	12600	71.5	13.8	4.8	5400	42.5	32.1	4.0	between	6600	47.3	30.3	5.0
21	25830	76.7	10.6	1.5	12480	81.2	9.2	6.2	between	14130	78.5	8.1	6.6
22	15000	85.1	6.8	4.4	6200	74.4	9.5	1.8	before	6200	74.4	9.5	1.8

WBC, white blood cell; SAGN, *Staphylococcus* infection-associated glomerulonephritis.

Phylogenetic analysis was performed independently (by authors LC and AW) and isolate strains were classified based on the following: (i) STs and CC, and (ii) by matching the isolate strains to complete reference genomes.[15-20] The closest reference genomes that matched were *S. aureus* JE2 (USA300), N315, and AR475. They did correspond exactly with the 3 CCs CC8, CC5 and CC59 respectively. Methods are described in the Supplementary Material. Pangenomic comparisons between the GN and non-GN samples was performed in the context of strain background only. The antimicrobial resistance genes were mined using AMRfinderPlus v3.10.5 whereas virulence genes were examined by Abricate v1.0.1 (https://github.com/tseemann/abricate) using the virulence factor database (http://www.mgc.ac.cn/VFs).[21] Antimicrobial resistance genes were identified by K-mer search or BLAST-like alignment tool from sources, including PATRIC, comprehensive antibiotic resistance database, and National database of antibiotic resistant organisms.[22,23] Genes present in all samples were filtered out and heatmaps were create2.

In vitro Functional Assays

Biofilm formation and production of toxins,[24-27] predominantly the beta-barrel pore-forming toxins such as alpha (α) and gamma (γ) hemolysins are key virulence factors of *S. aureus* particularly in patients with prosthetic joints and implantable devices.[26-28] Biofilm formation often leads to chronic wound infections with delayed wound healing.[29] Biofilms also skew the host immune response to an anti-inflammatory state that allow prolonged persistence of bacteria in the host.[30,31] We investigated the ability of the isolates for production of hemolysins and biofilms.

These assays were validated and performed as described below (and Supplementary Methods). Test isolates used (SAGN $n = 6$; non-GN $n = 12$) were same as for WGS. Control bacterial strains used are shown in Supplementary Table S2. All bacterial strains for the *in vitro* assays were grown in Tryptic Soy Broth (TSB) or Tryptic Soy Agar (TSA), with appropriate antibiotics (37 °C, liquid cultures shaken at 200 rpm), assay repeated 3 times.

Hemolysis Assay

To measure the β-hemolytic activity of the strains, overnight cultures from a single colony were diluted back and allowed to reach mid-log phase, normalized to optical density$_{600}$ = 0.5, followed by centrifugation of the cultures. The pellet was resuspended in Dulbecco's Phosphate Buffer Saline (DPBS) and 5 μl of the resuspended culture was spotted onto a blood agar plate (5% v/v defibrinated sheep's blood, 1.5% agar, 1% yeast extract, 1% tryptone, and 0.5% sodium

Table 2. Cases with archived blood culture isolatessite of infection, other comorbidities, renal function, urinalysis findings at the time of culture (or kidney biopsy for patients with GN) and at follow-up

S. aureus isolates	SAGN	WGS SeqName	Age	Sex	Race	DM type II	Type of infection	Other comorbidities	Blood culture	S. cr. at culture or biopsy	Urine protein	Urine RBCs per hpf	Follow-up S. cr.	Follow-up duration	Status at follow-up
SAGT-1	Absent	AG21-0302	63	M	C	Yes	Osteomyelitis	T9 paraplegia, neurogenic bladder	MRSA	0.4	Neg	1 to 2	0.3	4 years	Deceased
SAGT-2	Absent	AG21-0303	61	M	C	No	Bacteremia	RLL NSCLC, right thoracotomy, chylothorax	MRSA	0.7	Neg	1 to 2	1.03	7 years	Living
SAGT-3	Absent	AG21-0304	64	M	C	Yes	Mitral endocarditis	Valve abscess with perforation	MRSA	1.3	30 mg/dL	0 to 2	2.8	<1 year	Deceased
SAGT-4	Absent	AG21-0305	69	M	C	No	Cellulitis perineal	None	MRSA	1.3	Trace	3 to 5	1.3	<1 year	Living
SAGT-5	Absent	AG21-0306	40	F	C	Yes	Epidural abscess	IVDU, Hep C positive	MRSA	0.4	30 mg/dL	Trace	1.8	1 year	Living
SAGT-6	Absent	AG21-0307	74	M	C	No	Pneumonia	Esophageal stricture	MRSA	1.2	Neg	Absent	0.9	<1 year	Living
SAGT-7	Absent	AG21-0308	49	M	C	Yes	Pneumonia	MVA, cardiac arrest, acute encephalopathy	MRSA	0.5	Neg	0 to 2	0.4	<1 year	Deceased
SAGT-8	Absent	AG21-0309	68	M	C	No	Pneumonia	Brain diffuse large B-cell lymphoma	MRSA	0.5	30 mg/dL	3 to 5	0.3	<1 year	Deceased
SAGT-9	Absent	AG21-0310	48	F	C	No	Bacteremia	AML, chemotherapy, neutropenia fever	MRSA	1.4	100 mg/dL	0 to 2	0.7	2 years	Deceased
SAGT-10	Absent	AG21-0311	70	M	C	No	Pneumonia	None	MSSA	1.1	Neg	0 to 2	1.4	<1 year	Deceased
SAGT-11	Absent	AG21-0312	52	F	AA	No	Infected PICC line	Crohn's on TPN, enterocutaneous fistulas	MSSA	0.6	Neg	0 to 2	0.7	1 year	Deceased
SAGT-12	Absent	AG21-0313	30	M	C	No	Osteomyelitis, psoas abscess	GSW, paraplegia, DVT with filter	MRSA	0.4	Neg	0 to 2	0.7	<1 year	Living
SAGT-13	Present	AG21-034	60	M	C	Yes	Pneumonia	Prosthetic knee joint, morbid obesity	MRSA	6.1	100 mg/dL	>50	1.2	7 years	Living
SAGT-14	Present	AG21-0315	50	M	C	Yes	Sternal wound infection	None	MRSA	6	14 g/g	>20	2	<1 year	Living
SAGT-17	Present	AG21-0316	49	M	C	No	Tricuspid endocarditis	IVDU	MSSA	6.7	300 mg/dL	>20	5.1	1 year	Living; dialysis
SAGT-18	Present	AG21-0317	63	M	C	Yes	Septic arthritis	Urothelial cancer, cystectomy	MRSA	4.8	11.5 g/g	>20	3.8	1 year	Deceased
SAGT-20	Present	AG21-0318	61	M	C	No	Oral periapical abscess	None	MSSA	6.5	100 mg/dL	>20	0.7	<1 year	Living
SAGT-21	Present	AG21-0319	49	M	C	No	Septic arthritis	Enterocutaneous fistula, cavitary lung lesions	MSSA	1	Trace	20	0.8	<1 year	Deceased
SAGT-15	Present	No WGS	47	M	C	No	Multiple wounds	MVA	MRSA	1.9	1.2 g/g	>50	3.7	<1 year	Deceased
SAGT-16	Present	No WGS	25	M	C	No	Mitral, aortic endocarditis	IVDU, Hep C, also grew *Enterococcus faecalis*	MRSA	7.8	30 mg/dL	>20	0.8	1 year	Deceased
SAGT-19	Present	No WGS	39	F	C	No	Tricuspid endocarditis	IVDU, multiple abscesses, Hep C	MRSA	4.9	6.6 g/g	>20	4.8	<1 year	Living; dialysis

AG21, WGS sampling numbering; AML, acute myeloid leukemia; DVT, deep vein thrombosis; GSW, gunshot wound; Hep C, hepatitis C; hpf, high power field; IVDU, i.v. drug use; MRSA, methicillin-resistant *Staphylococcus aureus*; MSSA, methicillin-sensitive *Staphylococcus aureus*; MVA, motor vehicle accident; S. Cr., serum creatinine; SAGT, staphylococcal isolates; TPN, total parenteral nutrition; WGS, whole genome sequencing.

Table 3. Isolates mapped to sequence type, clonal complex and closest complete reference genomes after comprehensive genome analysis

Isolates	Date of culture	SeqName	Species	SAGN	ST	CC	SCCmec	*agr* type	*agr* mutants/WT	Variant calls *Staphylococcus aureus* JE2	Variant calls *S. aureus* N315	Variant calls *S. aureus* AR475
SAGT-1	9/10/2013	**AG21-0302**	MRSA	Absent	8	8	IV	I	WT	227	18,395	34,367
SAGT-2	12/18/12	**AG21-0303**	MRSA	Absent	105	5	II	II	WT	19260	1101	34,558
SAGT-3	1/3/2015	**AG21-0304**	MRSA	Absent	8	8	IV	I	WT	138	18,964	34,671
SAGT-4	1/12/2015	**AG21-0305**	MRSA	Absent	8	8	IV	I	WT	142	18,933	34,617
SAGT-5	1/30/2015	**AG21-0306**	MRSA	Absent	87	59	Not available	I	WT	34,680	34,853	157
SAGT-6	3/20/2014	**AG21-0307**	MRSA	Absent	8	8	IV	I	WT	94	18,948	34,645
SAGT-7	1/21/2016	**AG21-0308**	MRSA	Absent	8	8	IV	I	WT	163	18,951	34,679
SAGT-8	2/2/2016	**AG21-0309**	MRSA	Absent	8	8	IV	I	WT	137	18,925	34,629
SAGT-9	2/12/2016	**AG21-0310**	MRSA	Absent	105	5	IV	II	WT	19,389	1078	34,656
SAGT-10	6/7/2015	**AG21-0311**	MSSA	Absent	12	12	Not available	II	*agrC:* T70I	16,862	17,716	34,137
SAGT-11	6/21/2015	**AG21-0312**	MSSA	Absent	5	5	Not available	II	*agrC*:T70I	18,626	1023	34,083
SAGT-12	1/23/2016	**AG21-0313**	MRSA	Absent	87	59	II	I	WT	34,680	34537	251
SAGT-13	1/1/2013	**AG21-0314**	MRSA	Present	5	5	Not available	II	*agrB-agrA* del	18,823	1163	34,619
SAGT-14	1/13/2012	**AG21-0315**	MRSA	Present	5	5	II	II	*agrC*:G337D	19,638	720	34,802
SAGT-17	8/30/2016	**AG21-0316**	MSSA	Present	59	59	Not available	I	*agrC*: 128-135del frameshift	34,000	33,957	319
SAGT-18	10/7/2016	**AG21-0317**	MRSA	Present	105	5	II	II	*agrA*: G68D	18,875	912	34,214
SAGT-20	4/27/2015	**AG21-0318**	MSSA	Present	87	59	Not available	I	WT	34,622	34,480	239
SAGT-21	9/4/2015	**AG21-0319**	MSSA	Present	unique unidentified	none	II	I	*agrC* V26G,P242I, D246A,F293I	17,640	17,665	34,478

CC, clonal complex; GN, glomerulonephritis; JE2, N315, AR475, complete reference genomes; MRSA, methicillin-resistant Staphylococcus aureus; MSSA, methicillin-sensitive *Staphylococcus aureus*; SAGN, *Staphylococcus* infection-associated glomerulonephritis; ST, sequence type; WT, wild-type.
SAGT-10 did not cluster with any known complete reference genome; SAGT-21 did not belong to any known ST.

chloride). The plates were incubated at 37 °C with 5% carbon dioxide for 18 hours. The β-hemolytic activity was determined by calculating the ratio between the diameters of the zone of hemolysis and the spotted colony.[32,33]

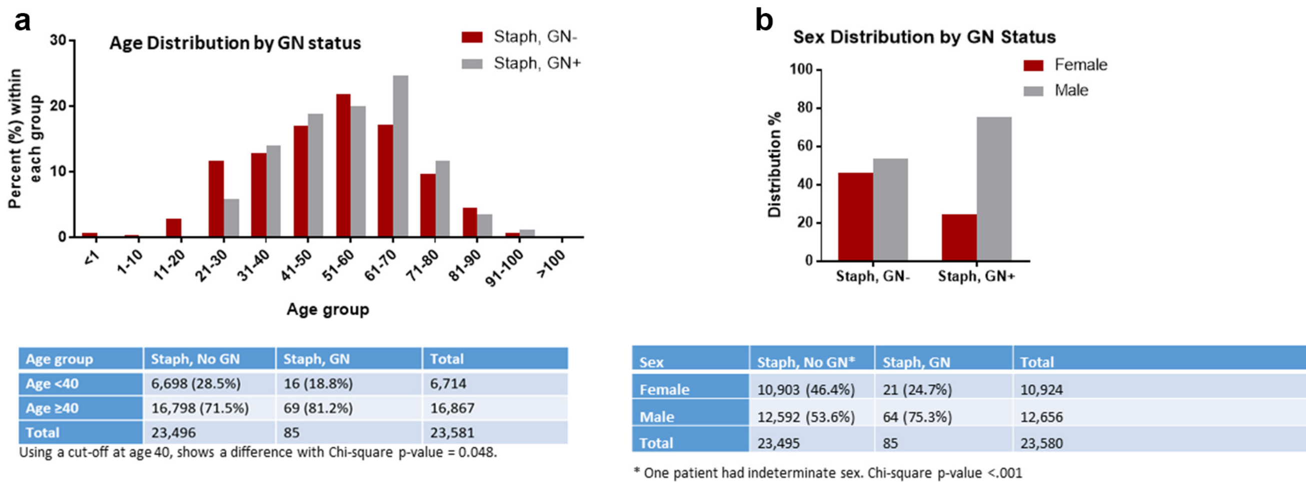
Biofilm Assay

Isolates were grown in Lysogeny Broth supplemented with 2% sodium chloride and 2% glucose. This medium showed consistent and reproducible results for quantifying *S. aureus* biofilms. Overnight cultures were diluted and grown to mid-log phase. In a 96-well flat-bottom plate, 150 μl of the normalized culture (optical density$_{600}$ = 0.5) was seeded and incubated for 4 hours (37 °C, static). The supernatant containing planktonic (free-floating) bacteria was carefully aspirated and the biofilms were gently washed 3 times with 1× DPBS. Biofilm biomass was quantified using crystal violet assay with slight modifications.[34] Briefly, the biofilms were stained with 0.1% crystal violet solution for 20 minutes at room temperature followed by 5 washes with DPBS. To elute crystal violet, 150 μl of 33% Glacial Acetic Acid was added and the plate was placed on a shaker for 20 minutes. The eluates were diluted 1:10 and optical density$_{590}$ was measured in a microplate reader. As controls, we used transposon mutants for *agrA* and *sarA* that upregulates biofilm-associated proteins for attachment and proteases for biofilm dispersal, respectively. Thus, *agrA*::Tn produces a strong biofilm while *sarA*::Tn does not form a biofilm (Supplementary Table S2).[35,36]

Detection of Poly-N-Acetylglucosamine

Poly-N-acetyl-β-(1-6)-glucosamine (PNAG), encoded by the *icaADBC* operon is a component of Staphylococcal biofilms and another key virulence factor.[37–40] The reason for performing this assay was related to the glomerular IgA containing immune complex deposits seen in SAGN, similar to those seen in primary IgA nephropathy.[6,7,41,42] We postulated that antibodies produced against Staphylococcal surface PNAG may cross-react with the exposed uncapped N-acetyl galactosamine residues in the hinge region of IgA molecules contributing to the pathogenic immune complexes. We investigated if isolates from patients with GN produced increased poly-N-acetylglu cosamine.

Immunoblot was perfomed with slight modifications.[43] Briefly, a lawn of an overnight culture from a single colony was grown on a TSA plate for 16 to 18 hours. The lawns were scraped and resuspended in 1



Age group	Staph, No GN	Staph, GN	Total
Age <40	6,698 (28.5%)	16 (18.8%)	6,714
Age ≥40	16,798 (71.5%)	69 (81.2%)	16,867
Total	23,496	85	23,581

Using a cut-off at age 40, shows a difference with Chi-square p-value = 0.048.

Sex	Staph, No GN*	Staph, GN	Total
Female	10,903 (46.4%)	21 (24.7%)	10,924
Male	12,592 (53.6%)	64 (75.3%)	12,656
Total	23,495	85	23,580

* One patient had indeterminate sex. Chi-square p-value <.001

Figure 2. (a) Age distribution by GN status. Overall distribution is similar for patients with and without glomerulonephritis, showing a higher prevalence of Staphylococcal infections in older adults. (b) Sex distribution by GN status. GN, glomerulonephritis.

ml of DPBS. The resuspended cultures were normalized to an optical density$_{600}$ of 30, centrifuged and resuspended in 100 μl of 0.5M EDTA (pH 8.0). They were boiled at 100 °C for 30 minutes with a brief vortex at the 15-minute mark. The boiled samples were centrifuged at 17,000 × g for 15 minutes. The supernatant was treated with Proteinase K (Ambion) at a final concentration of 0.5 mg/ml for 60 minutes at 65 °C, followed by deactivation for 5 minutes at 80 °C. On a nitrocellulose membrane, 2 μl of supernatant was spotted and allowed to air-dry for 10 minutes. The membrane was equilibrated in Tris-buffered saline (20 mM Tris-hydrochloric acid, 150 mM sodium chloride [pH 7.4]) with 0.5% Tween 20, blocked with 5% milk for 60 minutes, and incubated overnight with anti-poly-N-acetylglucosamine antibody (gift by Dr. Rajendar Deora) at a 1:1000 dilution. Bound antibodies were probed with a 1:5000 diluted rabbit anti-goat horseradish peroxidase-conjugated antibody (Bio-Rad, Hercules, CA) and detected using Amersham ECL Western Blotting Detection Reagents. The detected signals were quantified using ImageJ (National Institutes of Health, Bethesda, MD and Laboratory for Optical and Computational Instrumentation, University of Wisconsin).

Multilocus Sequence Typing and Pulsed-field Gel Electrophoresis

These were performed at the Southern Illinois University, School of Medicine, research laboratory of author MO (SAGN $n = 6$; non-SAGN $n = 12$) before beginning WGS.[44–47] Because they do not offer any additional information beyond that obtained by WGS, the results have been provided as Supplementary material (Supplementary Figures S1 and S2 and Supplementary Tables S3 and S4).

RESULTS

Host Demographic Features

Patient age and sex comparisons are shown in Figure 2. Both groups showed a similar age trend, but for patients with GN the largest number of patients were in the 61 to 70 years age group, which was higher compared with the non- patients with GN (Figure 2a). Using a cutoff at 40 years of age showed significant difference between the 2 groups (χ^2 $P = 0.048$). The male population was significantly larger in the GN group. (Figure 2b).

Laboratory Parameters

The median serum creatinine values were higher among the patients with GN as compared with patients without GN at both time points but not statistically significant (Figure 3a). There was a 0.3 mg/dl increase in median serum creatinine level in the GN group, indicating a worsening renal function among these patients, but this was also not statistically significant. The timing of the kidney biopsy in relation to the microbiological cultures was variable as can be expected in a retrospective study (Table 1), because it depended on several factors such as the clinical condition of the patient, and severity of renal symptoms. In addition, the temporal association of GN development relative to the onset of infection can vary from patient to patient. SAGN can occur anytime during the course of the infection but is frequently concurrent and without a definite infection-free latent period in between.[5–7] As a result, the timing of the kidney biopsy can also be variable relative to the timing of the microbial culture (as seen in our data; Table 1).

Total white blood cell counts, and differential lymphocyte and neutrophil counts did not show significant differences between the groups at either of the 2 time points (Figure 3b–d). Days to blood culture clearance

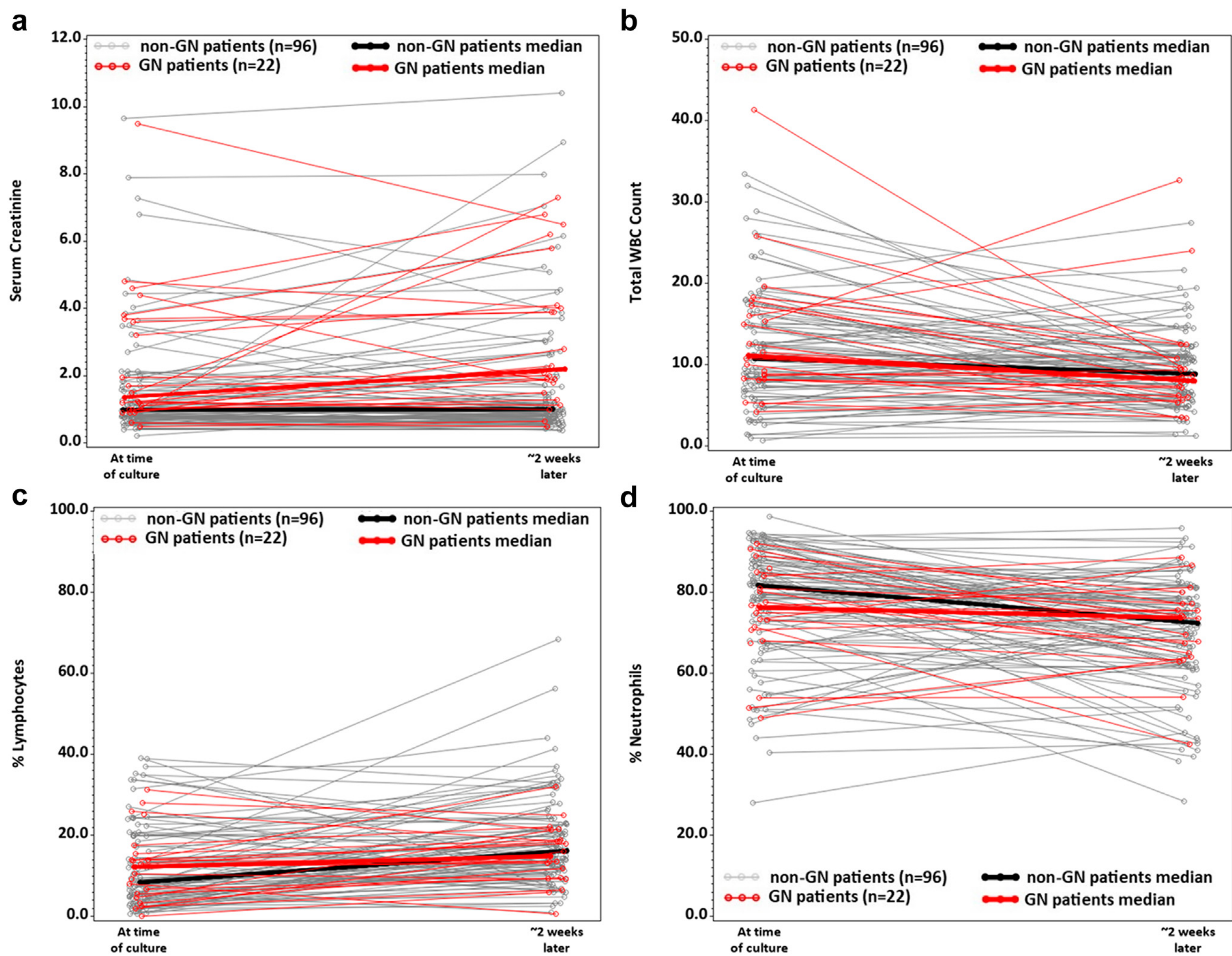


Figure 3. Comparison of laboratory parameters between GN ($n = 22$) and non-GN ($n = 96$) groups. (a) The median serum creatinine values were higher in the patients with GN as compared with patients without GN at both time points. There was an increase in median serum creatinine level by 0.3 mg/dl in the GN group, indicating a worsening renal function in these patients, but these were not statistically significant. (b) Total white blood cell counts, (c) differential lymphocyte, and (d) differential neutrophil counts did not show significant differences between the 2 groups at either of the 2 time points. GN, glomerulonephritis.

among the bacteremic patients (from first positive culture to first consecutive negative culture) showed a mean of 4 days for patients with GN and 3.4 days for non-GN (not statistically significant), so data is not shown.

WGS Reveals Multiple Strains in Both Groups

The isolates separated into multiple STs and CCs (Table 3). Among the 6 GN isolates, 3 belonged to CC5, 2 to CC59, and 1 isolate was a unique unidentified ST. In the non-GN control group, 6 clustered with CC8, 3 with CC5, 2 with CC59 and 1 with CC12. CC8 was not observed among the SAGN isolates, but this could be due to the small sample size. The CC assignments did correlate with the reference genome clustering as indicated by the variant calling (Table 3). Lower variant calls indicates greater similarity to the respective complete reference genome. The ST8 (CC8) non-GN isolates clustered with the JE2 reference genome. All 6 CC5 isolates clustered with N315, and CC59 isolates clustered with AR475. The single isolate (SAGT-10) from CC12 did not cluster with any known complete reference genome. Core SNP analysis showed that the 6 CC5 strains differed from each other with an average of 409 core SNPs (range: 78–718) and CC59 strains differed by an average of 228 core SNPs (range: 168–286), suggesting they are not the same strains, despite being within the same CC. These results suggest that the 6 GN isolates are heterogeneous and the infections in the patients with GN were not due to a clonal expansion of the same strain.

Mutations in *agr* Operon

Pan-genome analysis was performed for comparison of GN versus non-GN isolates, across similar CCs to avoid detecting lineage-specific differences (CC5 and CC59 respectively). CC8 isolates were compared with its own reference genome and the unidentified ST was compared with *agr* type I genome. One putative gene

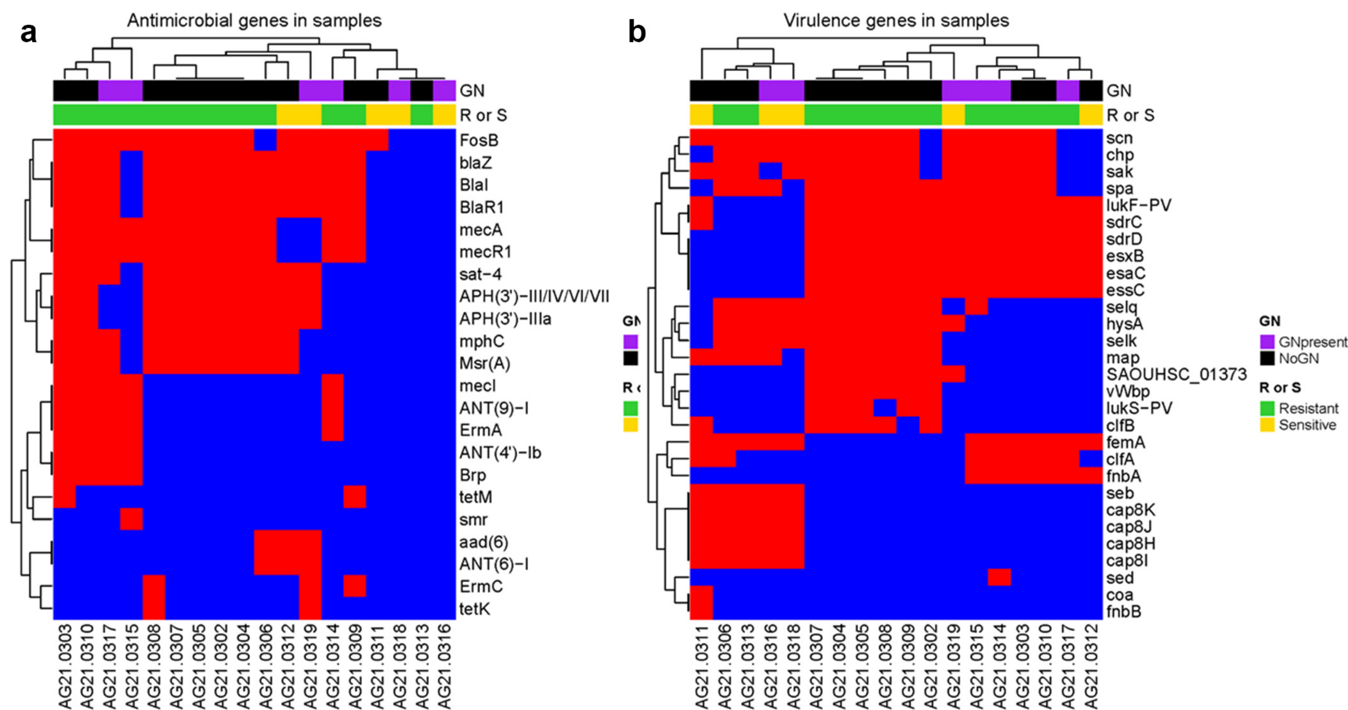


Figure 4. (a) Antimicrobial resistance genes were identified by K-mer search or BLAT from sources, including PATRIC, CARD, and NDARO. Red indicates the gene was detectable in the sample, blue indicates no gene was detected. (b) Virulence factors were identified by BLAT from sources VFDB or Victors. Genes present in all samples were filtered out since they were not informative. Red indicates the gene was detectable in the sample, blue indicates no gene was detected. VFDB, virulence factor database.

(aminoglycoside 3′ phosphotransferase, *aph*) was found to be present in the non-GN CC5 isolates but absent across the GN isolates.

SNP and InDel analysis showed mutations in the *agr* operon, detected mainly in the GN isolates (5 of 6) and only 2 of the 12 non-GN isolates. The *agr* mutations in each of the 5 GN isolates were mutually exclusive as follows: SAGT-13 (CC5) has 2848 base pair deletion encompassing *agrB* to *agrA* (*agrBDCA*); SAGT-17 (CC59) contains 7-base pair deletion in *agrC*, causing a frameshift and truncation of *agrC*; SAGT-14 carries single missense mutations in *agrC* (G337D) and SAGT-18 carries single missense mutation in *agrA* (G68D), both belonging to CC5; and SAGT-21 (unique unidentified ST) harbors multiple missense mutations in the *agrC* (V26G, P242I, D246A, and F293I). The 2 non-GN isolates share the same genetic changes (*agrC*: T70I). Details are shown in Table 3.

Antibiotic Resistance and Virulence Genes Show Only Lineage-Specific Differences

No specific differences were seen between the GN and non-GN isolates when compared across similar CCs (Figure. 4a and b). The apparent differences were mainly lineage specific.

In vitro Functional Assays

Reduced β-hemolytic Activity in GN Isolates

When compared with the wild-type strains, 5 of the 6 isolates from patients with GN (GN+) either exhibited a significant decrease or complete lack of β-hemolytic activity, which is also significantly different from the non-GN (GN−) isolates (Figure 5a). After obtaining WGS results on CC assignments of the isolates, comparison of GN + and GN- isolates within the respective CCs was also performed to eliminate strain-based lineage differences (Figure 5b). Grouped averages from the GN+ and GN- within each CC (CC5 and CC59) also showed significant difference (Figures 6a and b and 7a and b, respectively).

Increased Biofilm Forming Capacity in GN Isolates From CC5

Overall, no striking difference in biofilm mass between isolates from GN+ and GN− groups was observed (Supplementary Figure S3). Comparisons within the respective CCs was also performed for better specificity. Significantly higher biofilm formation was observed in the GN+ isolates than the GN− group within CC5 (Figure 6c and d), but not so in isolates from CC59 (Figure 7c and d).

No Significant Differences in Poly-N-acetylglucosamine (PNAG) production

Most isolates produced similar levels of PNAG as the WT USA300 (Supplementary Figure S4). Comparing GN+ and GN- isolates within CC5 and CC59 respectively, also did not show any significant differences (Figures 6e and f and 7e and f).

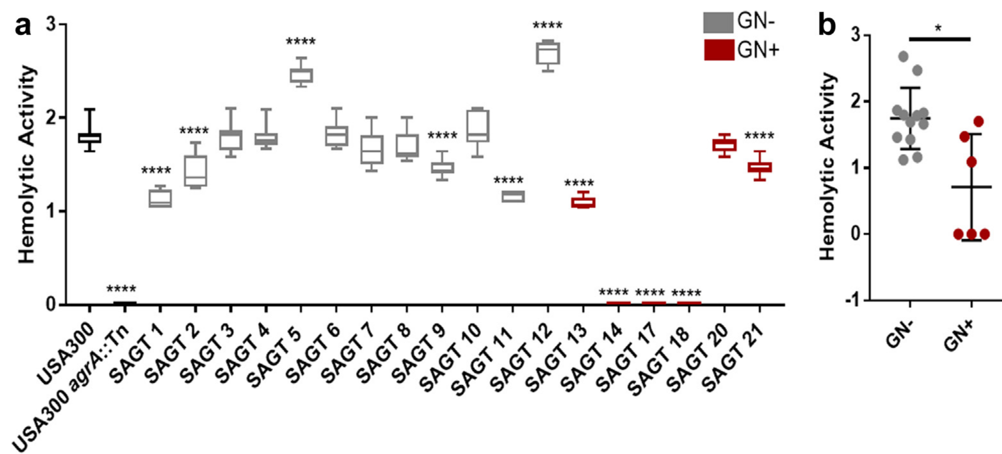


Figure 5. Reduced hemolytic activity in isolates from patients with GN. (a) *Staphyloccus aureus* isolates were spotted on 5% sheep blood agar to measure a zone of clearing due to lysis of erythrocytes. The cleared zone was measured along with the diameter of the spotted *S. aureus* colony to calculate hemolysis activity. Results obtained are from 3 independent experiments performed in triplicates. The data are represented as box and whiskers plot indicating the minimum and maximum values of hemolysis activity. (One-way ANOVA analysis, ****$P < 0.0001$). (b) Grouping averages of hemolytic activity of isolates from GN+ and GN- patients resulted in decreased hemolytic activity in isolates from GN+ patients compared with GN−. Each data point represents the average of results obtained from 3 independent experiment perfomed in triplicates. Data are presented as mean ± SD. (*$P < 0.05$. Unpaired *t* test with Welch's correction). ANOVA, analysis of variance; GN, glomerulonephritis.

DISCUSSION

Infection-related GN is an intriguing immunologic phenomenon characterized by the development of acute GN during or following the course of infection with pyogenic bacteria.[9] MRSA-associated GN is becoming the leading cause (along with methicillin-sensitive *S. aureus*), especially in the western subcontinents. This is a pilot study investigating predominantly microbial characteristics that may predispose some patients with infection to develop SAGN.

Genotyping of archived blood culture isolates from bacteremic patients, admitted at our hospital over the years was retrospectively performed. The GN isolates clustered into multiple CCs. No specific nephritogenic strains were identified (in contrast to post-streptococcal GN).[9] WGS revealed a higher incidence of *agr* mutations in the GN group (5 of 6 isolates, 83%) compared to the non-GN group (2 of 12, 16%). Many of the non-GN isolates were found to belong to CC8 (not seen among the GN isolates by WGS). This could be a bias of small sample size. The CC8 isolates, although appear to be similar (all *agr* type I), their time of collection was temporally far apart, and they were comparable to other isolates. In addition, the CC8 isolates were not included in the genomic comparison between the GN and non-GN isolates.

Agr quorum sensing is a pivotal mechanism controlling the vast armamentarium of virulence factors in *S. aureus*, ensuring appropriate expression and timely adaptation to changing microenvironments.[31-33] It exerts its effect through RNAIII driven promoters P2 and P3 for downstream target gene regulation, including several virulence genes.[48-52] The *agr* operon consists of 4 genes, namely *agrB, agrD, agrA, and agrC*.[48] The functional implications of the *agr* mutations in our isolates need further confirmation, however the *agr* gene deletion and truncation mutations identified in 2 of the GN isolates are likely to cause defects in *agr* functionalities. Another mutation we found (AgrA:G68D) has been shown to be lacking *agr* activity and δ-hemolysin production.[49] The overall decrease in β-hemolysis in the GN isolates and increased biofilm formation (demonstrated among the GN isolates of CC5) in our *in vitro* assays also correlates with the downstream effect of decreased *agr* activity in the GN isolates.[26] The *in vitro* assays were performed and validated (by authors PR and DW), independent of the WGS results. We did not find significant differences in PNAG levels between the GN and non-GN isolates. Nevertheless, our hypothesis that antibodies produced against Staphylococcal surface PNAG may cross-react with the exposed uncapped N-acetyl galactosamine residues in the hinge region of IgA molecules contributing to the pathogenic immune complexes seen in SAGN needs to be investigated using a better experimental model.

A significant fraction of *S. aureus* bacteremia cases have previously been shown to be associated with mutations in 1 or more of the *agr* genes.[53,54] Traber *et al*[48] have demonstrated *agr*-defective mutants even in fresh clinical samples with a minimum of post-isolation manipulation, proving that these mutations do arise *in vivo* and not due to post-isolation handling *in vitro*.

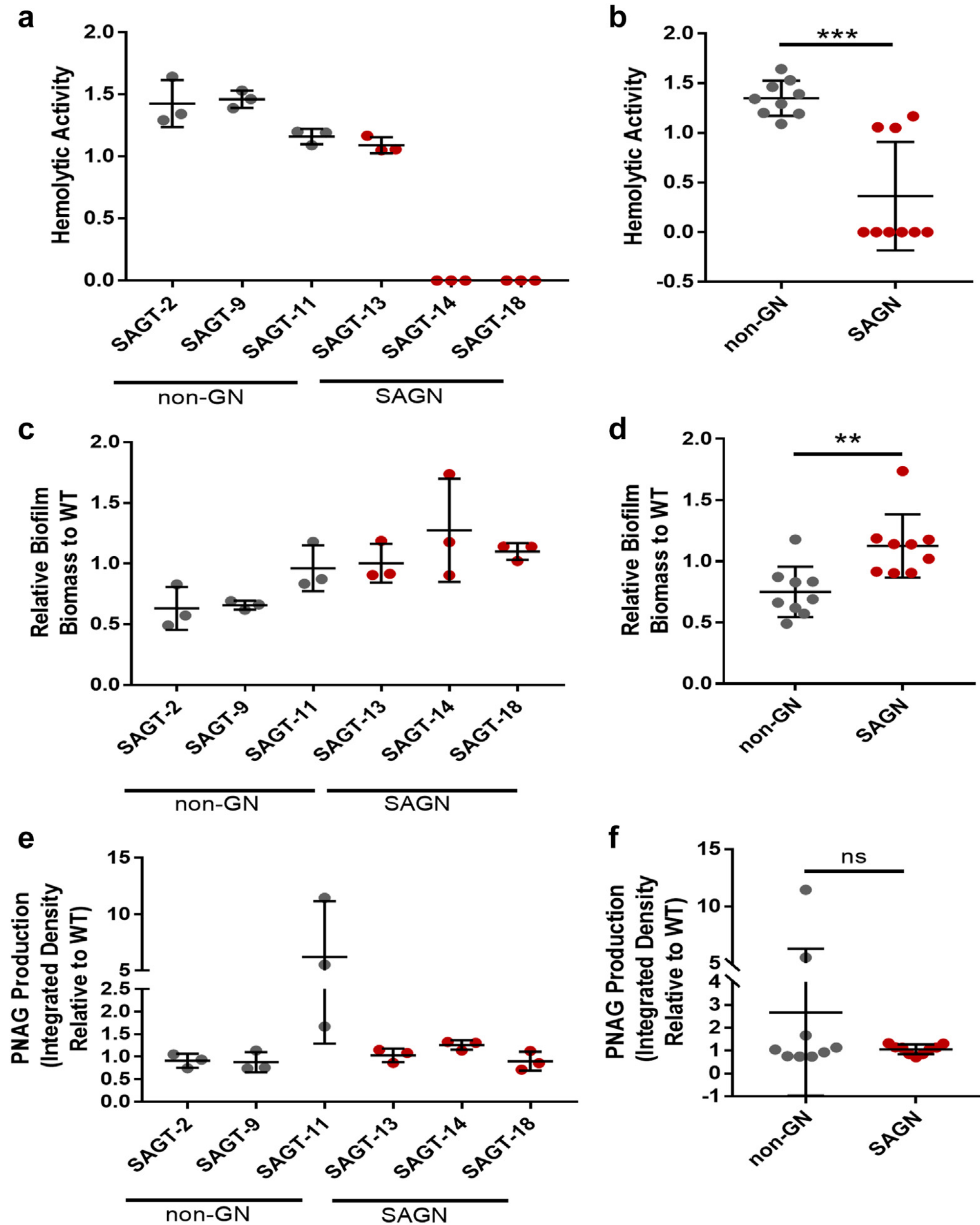


Figure 6. Comparative analyses on hemolysis, biofilm formation, and PNAG production between GN+ and GN− isolates within CC5 clonal complex. Data obtained from *in vitro* (a) hemolysis, (c) biofilm, and (e) PNAG production assays were grouped as isolates from GN- (gray) and GN+ (red) within CC5. The averages for each group were taken for comparison. On average, isolates from GN+ group within CC5 exhibited (b) decreased hemolytic activity and (d) increased biofilm production, while there was no significant difference in (f) PNAG production. Results represent an average of 3 independent experiments performed in triplicates. Data are presented as mean ± SD. (**$P < 0.01$, P ***<0.001. Unpaired t test with Welch's correction). CC, clonal complex; GN, glomerulonephritis; ns, not significant; PNAG, Poly-N-acetyl-β-(1-6)-glucosamine; SAGN, *Staphylococcus* infection-associated glomerulonephritis.

Historically, mutations in the *agr* operon predominantly found in *agrC* or *agrA* are typically found to be inactivating the autoactivation circuit and thereby reducing expression from RNAIII.[52] These mutations were associated with decreased exotoxin production, but concomitant increase in surface-associated adhesin factors (such as fibronectin-binding proteins [FnbA, FnbB]) needed for initial attachment, conferring a survival advantage to the microbe in certain host niches, particularly while in the blood stream early in the course of infection when bacterial numbers are low.[50,51,53] Lack of toxin production results in decreased host inflammatory responses and evasion from host defenses leading to enhanced survival in the blood stream. The *agr* quorum sensing system can also co-ordinate the switch from expression of surface-associated adhesin factors needed for initial attachment, to the expression of exotoxins produced when a high bacterial cell density is

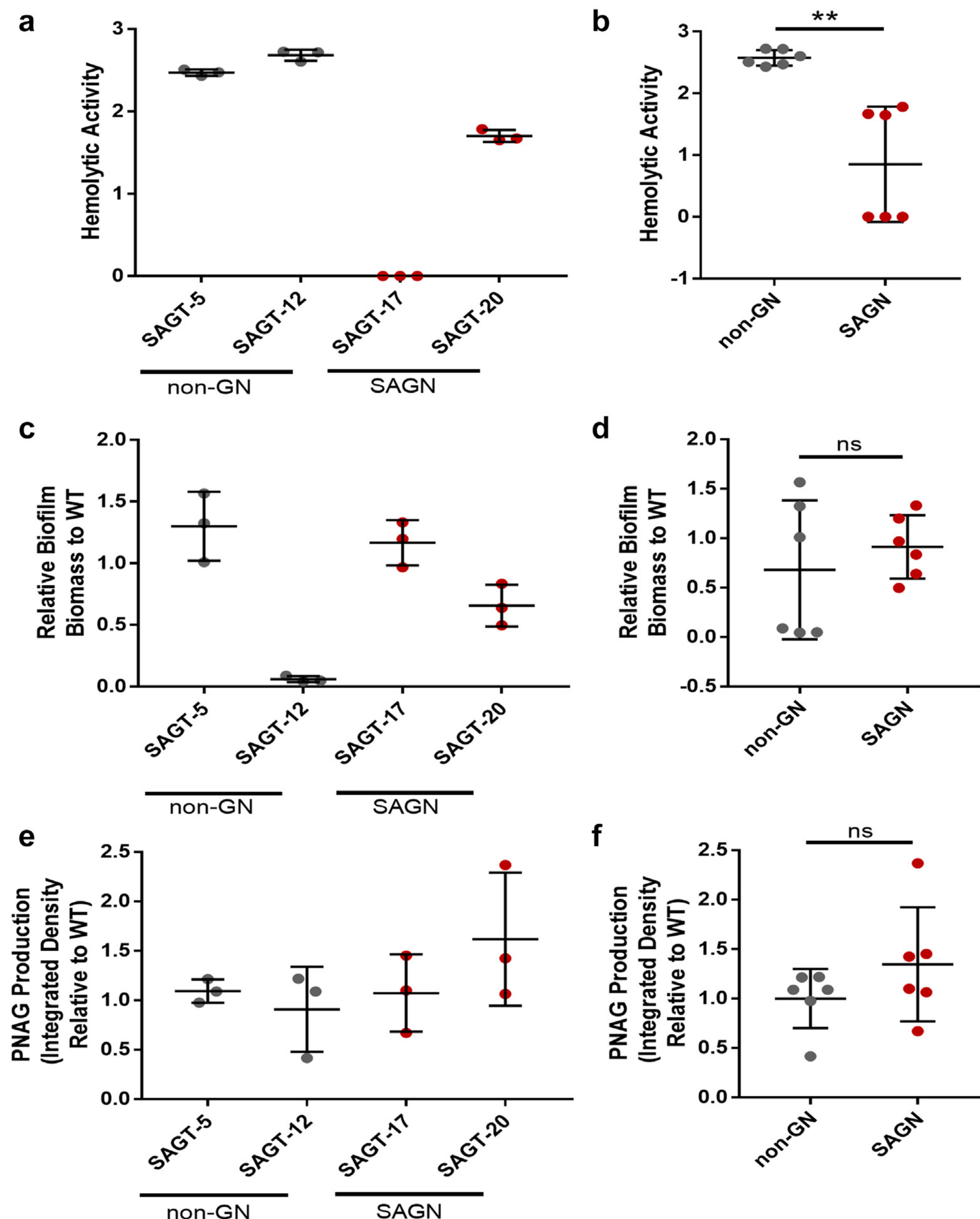


Figure 7. Comparative analyses on hemolysis, biofilm formation, and PNAG production between GN+ and GN− isolates within CC59 clonal complex. Data obtained from *in vitro* (a) hemolysis, (c) biofilm, and (e) PNAG production assays were grouped as isolates from GN- (gray) and GN+ (red) within CC5. The averages for each group were taken for comparison. On average, isolates from GN+ group within CC5 exhibited (b) decreased hemolytic activity, while there was no significant difference in (d) biofilm and (f) PNAG production. Results represent an average of 3 independent experiments performed in triplicates. Data are presented as mean ± SD. (**$P < 0.01$. Unpaired *t* test with Welch's correction). CC, clonal complex; GN, glomerulonephritis; ns, not significant; PNAG, Poly-N-acetyl-β-(1-6)-glucosamine; SAGN, *Staphylococcus* infection-associated glomerulonephritis.

established in the host tissue.[49] Other reported effects of reduced *agr* activity include increased expression of the immune evasion molecule Staphylococcal protein A (spa) and *fnbA*,[54] and increased expression of superantigen-like proteins that modulate host immune functions.[55-57] FnbA adhesin promotes invasion of and persistence of the bacteria within endothelial cells, important in deep-seated tissue infections (like endocarditis, osteomyelitis, visceral abscesses), reaching a niche beyond the reach of immune surveillance mechanisms, and some antibiotics like vancomycin which penetrates poorly within endothelial cells.[34,40] Such tissue infections are common in patients with SAGN.[1,6-8]

Large systematic meta-analyses have shown dysfunctional *agr* to be associated with unfavorable outcomes in invasive MRSA infections.[58] Our study cohort is too small to compare outcomes. Site and severity of infection, and promptness in initiating treatment probably also are likely to play an equally important role in outcomes. A study comparing

S. aureus blood stream isolates from patients with persistent bacteremia (>7 days on adequate antibiotic treatment) and patients with resolved bacteremia (<4 days on adequate antibiotic treatment) showed more frequent defects in production of δ-lysin, a surrogate marker for *agr* locus dysfunction.[51] In our study, days to blood culture clearance (from first positive culture to first consecutive negative culture) among the bacteremic patients showed a mean of 4 days for patients with GN and 3.4 days for non-GN (not statistically significant), and so data is not shown.

Among host factors, males stood out in our SAGN cohort. Advanced patient age appears to be a risk factor for *S. aureus* infections in general, but not specifically for SAGN. Routine laboratory parameters do not appear to be useful to predict development of SAGN, emphasizing the need for genomic markers.

The small sample size is the main pitfall of this study. Obtaining archived culture samples from human patients can present several challenges as follows: (i) The number of proven patients with SAGN in any single center is low; (ii) Patients with SAGN present at random, the disease is not recurrent and periodic planned patient encounters do not occur as in relapsing glomerulonephritides such as lupus nephritis; and (iii) Laboratory regulations, preclude storage of large number of isolates and also isolates from sites other than blood. Despite these limitations, this pilot study is a unique and a much-needed attempt at making some headway into this enigmatic kidney disease.

The much higher prevalence of *agr* mutations in our GN isolates compared with the non-GN isolates is the key finding in this study and provides useful insights, although further elucidation in terms of *agr* functionality is needed. Nevertheless, based on published work in this area of study, prolonged bacteremia, secondary seeding of infection, and chronicity of infection facilitated by reduced *agr* activity could be important factors predisposing to development of SAGN. Our findings have important implications in light of the considerable interest in development of therapeutic agr inhibitors as novel antivirulence drugs.[59,60] Moreover, testing for *agr* mutations early in infection could potentially be useful to predict risk for SAGN. Larger multi-institutional studies are much needed.

DISCLOSURE

All the authors declared no competing interests.

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SUPPLEMENTARY MATERIAL

Supplementary File (PDF).

Supplementary Methods description

Figure S1. Burst diagram grouping the major sequence types identified on the MLST study, based on mutations in 7 genes.

Figure S2. Compilation of PFGE gels of the tested isolates.

Figure S3. Biofilm forming capacity does not show significant difference between GN+ and GN- isolates overall.

Figure S4. PNAG production by *S. aureus* does not differ significantly between GN+ and GN- isolates.

Table S1. Demographic profile of the GN and non -GN cohorts used for comparison of laboratory parameters.

Table S2. Control bacterial strains for *in vitro* functional assays.

Table S3. Primer list for seven genes used for MLST.

Table S4. MLST results showing amplified genes, sequencing data, and derived sequence types and clonal complexes of the GN and non -GN isolates using eBURST, a Web -implemented clustering algorithm (https://pubmlst.org/organisms/staphylococcus -aureus.

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