

# $\alpha$ -Hemolysin, Not Panton-Valentine Leukocidin, Impacts Rabbit Mortality From Severe Sepsis With Methicillin-Resistant *Staphylococcus aureus* Osteomyelitis

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**Background.** Severe sepsis, combining acute osteomyelitis and lung involvement, has been described increasingly in healthy children with the spread of community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA).

**Methods.** Outcomes (mortality, hematogenous spread, lung and bone involvements) of rabbit osteomyelitis caused by CA-MRSA LAC<sup>WT</sup> USA300 and its Panton-Valentine leukocidin (PVL)- and  $\alpha$ -hemolysin (Hla)-negative isogenic derivatives (LAC $\Delta$ pvl and LAC $\Delta$ hla, respectively) were compared.

**Results.** Three days after inoculation (D3), all LAC<sup>WT</sup>- and LAC $\Delta$ pvl-, and 72% of LAC $\Delta$ hla-infected rabbits had no hematogenous spread and similar lung and bone bacterial densities. LAC $\Delta$ pvl and LAC $\Delta$ hla caused less severe histological lung lesions than LAC<sup>WT</sup> ( $P \leq .01$ ). Between D3 and D9, 10 (53%) LAC<sup>WT</sup>-, 11 (55%) LAC $\Delta$ pvl-, but no LAC $\Delta$ hla-infected rabbits ( $P < .005$ ) died of severe sepsis with disseminated infection. Unlike deceased animals, most LAC<sup>WT</sup>, LAC $\Delta$ pvl, and LAC $\Delta$ hla D14 survivors had no hematogenous spread ( $P < .001$ ). LAC<sup>WT</sup> (88%) caused more bone abscesses than LAC $\Delta$ pvl (0,  $P = .001$ ) or LAC $\Delta$ hla (30%,  $P = .01$ ).

**Conclusion.** In this model, both PVL and Hla seemed to be required for early lung involvement via hematogenous spread. Hla, but not PVL, significantly impacted severe sepsis-related mortality. PVL was the predominant factor determining late-stage bone abscesses.

**Keywords.** *Staphylococcus aureus*; CA-MRSA; osteomyelitis; severe sepsis; Panton-Valentine leukocidin;  $\alpha$ -hemolysin; rabbit model.

Acute hematogenous osteomyelitis has long been recognized as a major clinical manifestation of *Staphylococcus aureus* diseases in children [1]. As Panton and

Valentine wrote, even before the antibiotic era, “. . . in most of the cases despite the high frequency of bacteraemia, metastatic infection is infrequently encountered and mortality rates are very low, however, there are cases with a positive blood culture in which the prognosis is obviously grave” [2]. They suspected a role of Panton-Valentine leukocidin (PVL) in the course of osteomyelitis and reported a relationship between infection severity and anti-leukocidin antibody titers in the sera of some patients during the acute phase of the disease [3]. However, at that time, molecular characterization of the strains could not be performed.

The community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) era has renewed

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interest in severe sepsis associated with acute osteomyelitis (AOM). Since CA-MRSA developed and spread rapidly in the United States, severe invasive staphylococcal infections in healthy children and adolescents have been increasingly described. The alert was first raised by the Centers for Disease Control and Prevention [4] and then by pediatricians in Texas [5], who reported, within 2 years, 14 adolescents with severe staphylococcal sepsis combining pulmonary involvement with bone-and-joint infections. Genes encoding for PVL were present in all isolates, including the 2 methicillin-sensitive *S. aureus* strains [5], suggesting this toxin played a role in the clinical presentation. The question of PVL's contribution to symptom severity was further heightened by clinical studies showing that pediatric PVL-positive staphylococcal osteomyelitis was significantly associated with local complications, for example, myositis, pyomyositis, and venous thrombosis, and also with severe sepsis and lung abscesses [6–8]. *S. aureus* hematogenous spread to the lung associated with osteomyelitis in children frequently appeared to be revealed by abscesses, empyemata, or septic emboli [8, 9] and to have a lower case-fatality rate than airborne-transmitted necrotizing pneumonia [10]. A recently published meta-analysis concluded that, even though musculoskeletal infections caused by PVL strains are much less common than skin and soft-tissue infections, children with PVL-positive bone, muscle, and joint infections might have more morbidity than those infected by PVL-negative strains [11].

In addition to PVL, CA-MRSA, especially USA300, has been shown to overexpress a number of core-genome-encoded virulence factors, such as  $\alpha$ -hemolysin (Hla) and phenol-soluble modulins [12]. Hla is a critical virulence factor in numerous animal infection models, for example, brain abscesses [13], skin and soft-tissue infections [14], and pneumonia [15]; thus, it could contribute to the observed systemic severity of CA-MRSA AOM.

An experimental model closely reproducing the human situation is essential to explore the pathophysiology of bacterial infections, especially for bacteria generating a broad spectrum of clinical manifestations. We previously showed that PVL production in experimental CA-MRSA USA300 AOM in rabbits was associated with extraosseous infection extensions, specifically muscle abscesses and prolonged systemic inflammation [16], consistent with clinical observations in children [6]. However, the respective roles of PVL and Hla on sepsis severity and pulmonary hematogenous spreading have never been studied in experimental AOM.

The goals of this study were to evaluate PVL and Hla impacts on mortality, hematogenous spread, and lung and bone involvements in a rabbit AOM model, by comparing the outcomes of infections caused by LAC<sup>WT</sup> USA300 MRSA and its PVL- and Hla-negative isogenic derivatives (LAC $\Delta$ pvl and LAC $\Delta$ hla, respectively).

## MATERIALS AND METHODS

### Bacterial Strains

We used a clinical *S. aureus* strain belonging to the USA300 lineage (LAC<sup>WT</sup>), and its isogenic  $\Delta$ lukS/F-PV and  $\Delta$ hla derivatives (forming LAC $\Delta$ pvl and LAC $\Delta$ hla, respectively), all kindly provided by Frank DeLeo [15, 17]. Specific PVL and Hla enzyme-linked immunosorbent assays (ELISA) were used to verify PVL and Hla production in the supernatants of the 3 strains, as described elsewhere [18, 19].

### Preparation of Bacterial Inocula

The microorganisms were stored at  $-80^{\circ}\text{C}$  until use. Prior to experiments, they were cultured in casein hydrolysate and yeast-extract medium (CCY) at  $37^{\circ}\text{C}$  for 18 hours with shaking. After centrifugation, the pellets were washed and resuspended in phosphate-buffered saline (PBS) and quantitatively cultured.

### Experimental Osteomyelitis in Rabbits

Norden's method [20] was used to induce osteomyelitis in female New Zealand white rabbits weighing 2–3 kg. The experimental protocol complied with French legislation on animal experimentation and was approved by the Animal Use Committee of Maison Alfort Veterinary School. The animals were anesthetized with intramuscularly injected ketamine (25 mg/kg; Vibrac, France) and 2% Xylazine Rompum (25 mg/kg; Bayer Santé, Division Santé Animal). Before *S. aureus* challenge (on day (D) 0), 500  $\mu\text{L}$  of venous blood were drawn for determination of anti-PVL and anti-Hla antibody titers and C-reactive protein (CRP) concentrations. Infection was induced by tibial injection of a sclerosing agent (Trombovar<sup>®</sup>), followed by  $4 \times 10^8$  CA-MRSA colony-forming units (CFU) in 0.2 mL, and by 0.1 mL of saline. Patch analgesia (Durogesic<sup>®</sup>) was given for 7 days following surgery. Twenty-four hours after bacterial challenge, blood samples from 12 animals given each strain were cultured to detect transient bacteremia.

### Animal Groups

Rabbits were assigned to receive  $4 \times 10^8$  CFU of LAC<sup>WT</sup> (n = 24), LAC $\Delta$ pvl (n = 26) or LAC $\Delta$ hla (n = 19). Five LAC<sup>WT</sup>-, 6 LAC $\Delta$ pvl-, and 7 LAC $\Delta$ hla-infected animals were killed 3 days postinoculum (D3). Nineteen LAC<sup>WT</sup>-, 20 LAC $\Delta$ pvl-, and 12 LAC $\Delta$ hla-infected rabbits were observed until D14. Six uninfected animals served as controls.

### Macroscopic Appearance and Sample Collection

The animals were monitored daily for general and local signs of infection (mobility, leg appearance). Moribund animals (immobile, unable to be aroused from a recumbent position and/or unable to access food and water) were killed by rapid intravenous pentobarbital injection [21].

Before death, venous blood was drawn for cultures and serum samples. On the day of death, lungs, right leg, spleen, and

kidneys were removed. Both lungs were weighed and visually examined. Presence of congestion and any color modification were recorded. The right leg was examined: presence and location of purulent exudates in the joint space, soft-tissue and bone abscesses, and tibial metaphysis deformation were noted. Photographs taken to document the macroscopic appearance of lungs and right tibia were evaluated by a blinded investigator, unaware of study-group attribution, and yielded observations comparable to and consistent with autopsy findings.

Right lungs were collected for histological examination, and left lungs were stored at  $-80^{\circ}\text{C}$  for determination of bacterial densities, PVL, and Hla concentrations. Infected tibias were removed. The upper third of the tibia was frozen in liquid nitrogen, crushed in a pulverizer (Spex 6700; Freezer/Mill Industries), suspended in 10 mL of sterile saline and quantitatively cultured on tryptic soy agar. When present, subcutaneous abscess fluids were stored at  $-80^{\circ}\text{C}$  for determination of PVL concentrations. Spleen and kidneys were crushed and cultured on blood agar.

### Histological Examination

Immediately after death, the right lung was fixed in 10% formaldehyde. Forty-eight hours later, a sample of each lobe was embedded in paraffin. Slides of hematoxylin-and-eosin-stained, 5- $\mu\text{m}$ -thick sections were reviewed by a pathologist blinded to study-group attribution. For each lung specimen, the following parameters were scored (0–4: none, minimal, mild, moderate, severe): congestion, inflammation, megakaryocytes, abscesses, infarcts, thrombi, pleural involvement, and presence of bacteria.

### Determination of PVL and Hla Concentrations

PVL and Hla concentrations in rabbit samples were determined with previously described specific ELISA [18, 19] using specific antibodies kindly provided, respectively, by bioMerieux R&D Immunodiagnostic (France) and MedImmune Inc. (USA).

### Serum-Antibody Assay

Before *S. aureus* challenge and at death, the presence of anti-PVL antibodies was assessed with a specific ELISA [16]. For some animals, antibodies directed against Hla were also evaluated with a specific ELISA using recombinant Hla (in-house), Nabi® rabbit anti-Hla polyclonal antibody (for the standard range), and peroxidase-conjugated swine anti-rabbit polyvalent immunoglobulin G (IgG; Dako). The results are expressed in arbitrary units/milliliter (AU/mL), with 1 AU corresponding to the amount of anti-Hla antibodies contained in a  $1/10^6$  dilution of the reference polyclonal rabbit serum.

Serum CRP was assayed with a specific ELISA, according to the manufacturer's instructions (Eurobio).

### Statistical Analyses

Percentages of hematogenous spread (positive blood, spleen, or kidney cultures), bone abscesses and deformations, and infected

lungs were compared with Fisher exact test. The nonparametric Mann–Whitney *U*-test was used to compare bacterial counts, PVL and Hla lung concentrations, anti-PVL and anti-Hla antibody titers, CRP levels, and histological scores. Probability of progression to death was calculated with the Kaplan–Meier method, with inoculation as time 0 and censoring at death. Survival distributions were compared with the log-rank test.  $P < .05$  defined significance.

## RESULTS

### Characteristics of Experimental LAC<sup>WT</sup> AOM

Among the 24 rabbits given the  $4 \times 10^8$  CFU LAC<sup>WT</sup> inoculum, 5 were killed on D3 to evaluate lung and bone lesions at the early disease stage (see Table 1). Ten died between D3 and D9 (mean: D4.3) of severe sepsis with disseminated infection. The 9 survivors were killed on D14.

D1 blood cultures were positive for half the rabbits tested (6/12; including 3 in the group of 6 rabbits that died and 3 in the group of 6 rabbits that survived), revealing no correlation between positive blood culture and early mortality, whereas blood, spleen, and kidney cultures from all animals killed on D3 were sterile.

Early-stage (D3) findings showed hematogenous lung lesions. In most rabbits (3/5, 60%), the lungs appeared red and congestive and were infected with a median bacterial density of  $6.2 \log_{10}$  CFU/g of tissue. Histological examinations found lung congestion and inflammation, abscesses, thrombi, infarcts, and pleural involvement. Histological lung scores are reported in Figure 1. All rabbits had infected bones (median bacterial density:  $5.8 \log_{10}$  CFU/g) with microabscesses at the inoculation site but still no cortical deformation. CRP concentrations were elevated compared to baseline (data not shown). PVL was detected in lung and bone abscesses, whereas Hla was detectable in the lung of only 1 rabbit on D3.

Between D3 and D9, half of the LAC<sup>WT</sup>-infected animals died of severe sepsis with weight loss (mean: 50%), bacteremia (all kidney and spleen cultures positive) and hematogenous lung lesions. All deceased animals' lungs were infected with high bacterial densities (median:  $8.2 \log_{10}$  CFU/g of lung) and contained high PVL (median: 37.7 ng/g of lung) and Hla concentrations (median: 244 ng/g of lung). Macroscopic aspects and histological lung scores (Figure 1) were similar to those observed on D3 (nonsignificant [NS]). Typical examples of lung macroscopic and histological lesions are shown in Figure 2. All rabbits' bones were also infected (median:  $6.4 \log_{10}$  CFU/g) with no sign of local severity (20% had microabscesses but no deformation).

Unlike nonsurvivors, D14 survivors had no signs of bacteremia/severe sepsis but had extensive bone lesions. Blood, kidney, and spleen cultures were sterile for 90% of the rabbits ( $P < .001$  vs nonsurvivors). Significantly fewer LAC<sup>WT</sup>-infected survivors

**Table 1. Macroscopic and Microbiological Findings and Toxin Concentrations at Necropsy**

Rabbits	Strain (n Rabbits)	Hematogenous Spread <sup>a</sup>	Bone				Lung			
			Abscess	Deformation	Median Log <sub>10</sub> CFU/g	Median PVL in Abscesses (ng/g)	% Infected	Median Log <sub>10</sub> CFU/g	Median PVL (ng/g)	Median Hla (ng/g)
Killed D3	LAC <sup>WT</sup> (5)	0%	100% <sup>b</sup>	0%	5.8	71	60%	6.2	6.8	0
	LACΔpvl (6)	0% [NS] <sup>c</sup>	66% <sup>b</sup> [NS]	0% [NS]	6.8 [NS]	...	83% [NS]	4.2 [NS]	...	0 [NS]
	LACΔhla (7)	28% [NS]	14% <sup>b</sup> [.015]	0% [NS]	5.3 [NS]	...	57% [NS]	2.55 [NS]	0 [.007]	...
Died D3–D9 <sup>d</sup>	LAC <sup>WT</sup> (10)	100%	20%	0%	6.43	...	100%	8.19	37.7	244
	LACΔpvl (11)	100% [NS]	9% [NS]	0% [NS]	6.98 [NS]	...	100% [NS]	7.2 [NS]	...	34 [NS]
	LACΔhla (2)	100% [NS]	0% [NS]	0 [NS]	6.03 [NS]	...	100% [NS]	8.44 [NS]	1995 [NS]	...
Killed D14	LAC <sup>WT</sup> (9)	11%	88%	66%	6.15	...	28%	2.1	0	0
	LACΔpvl (9)	22% [NS]	0% [.001]	33% [NS]	3.9 [NS]	...	43% [NS]	2.01 [NS]	...	0 [NS]
	LACΔhla (10)	20% [NS]	30% [.01]	10% [NS]	4.4 [NS]	...	40% [NS]	1.85 [NS]	0 [NS]	...

Abbreviations: CFU, colony-forming units; Hla, α-hemolysin; NS, not significant; PVL, Pantone-Valentine leukocidin.

<sup>a</sup> Defined as positive culture(s) of blood, spleen, or kidney.

<sup>b</sup> Microabscesses at the injection site.

<sup>c</sup> P values vs LAC<sup>WT</sup> appear in brackets. P < .05 defined significance.

<sup>d</sup> No LACΔhla-infected rabbits died between D3 and D9; 1 each died on D11 or D14.

vs nonsurvivors, respectively, also had infected lung involvement (28% vs 100%,  $P < .021$ ), fewer lung CFU (median: 2.1 vs 8.1,  $P = .003$ ), lower PVL (0 vs 37.7 ng/g,  $P = .001$ ) and Hla concentrations and (0 vs 244 ng/g, NS), and less severe histological lesions (megakaryocytes  $P = .01$ , abscesses  $P = .02$ , thrombi  $P = .005$ ). In contrast, local extension of the bone infection was visible in the majority of animals: 88% had abscesses and 66% bone deformations. These severe bone lesions were associated with high anti-PVL and anti-Hla antibody titers in convalescent animals (Figure 3).

#### Comparison of LAC<sup>WT</sup> vs LACΔpvl Experimental AOM (Table 1)

Among the 26 rabbits given the  $4 \times 10^8$  CFU LACΔpvl inoculum, 6 were killed on D3; 11 died of severe sepsis with disseminated infection between D3 and D9; and the survivors were killed on D14.

At the early stage (D3), mean bacterial counts in lungs and bones were similar for LAC<sup>WT</sup> and LACΔpvl. However, LACΔpvl-infected rabbits had significantly less severe histological lung lesions than LAC<sup>WT</sup>-infected animals: inflammation ( $P = .004$ ), lung abscesses ( $P = .011$ ), pleural involvement ( $P = .002$ ), megakaryocytes ( $P = .01$ ), and bacteria ( $P = .011$ ) but not thrombi (NS) (Figure 1). As for LAC<sup>WT</sup>-infected rabbits, Hla was not detectable in lung on D3.

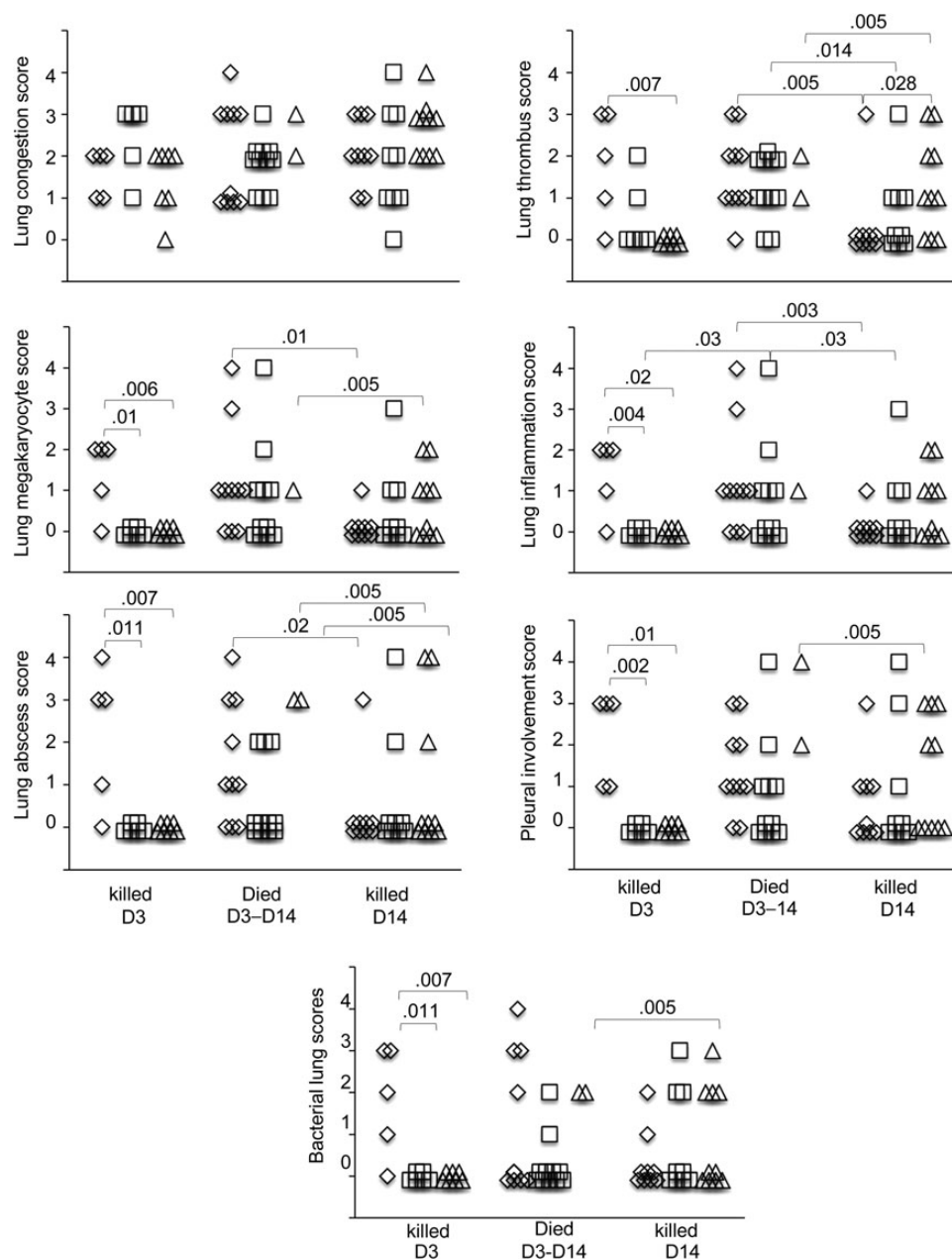
Between D3 and D9, the survival rate (Figure 4), percentage of dead animals, weight loss at the time of death (data not shown), bacterial densities in lungs and bones of dead animals, lung histological scores, and bone lesions were similar for LAC<sup>WT</sup>- and LACΔpvl-infected rabbits. The Hla density in LACΔpvl lungs (34 ng/g) was lower than in LAC<sup>WT</sup>-infected rabbits but not significantly so.

In D14 survivors, bone infections persisted with both strains but bone abscesses, the hallmark of PVL, were significantly more frequent in LAC<sup>WT</sup>- than LACΔpvl-infected rabbits (88% vs 0%, respectively;  $P = .001$ ). These severe LAC<sup>WT</sup> bone lesions were associated with higher CRP levels (240 vs 85 μg/mL,  $P = .002$ ) and anti-PVL antibody titers vs LACΔpvl (22 000 vs 4000 AU/mL,  $P = .017$ ) (Figure 3). Unexpectedly, LACΔpvl-infected rabbits had significantly higher anti-Hla antibodies (48 300 vs 24 000 AU/mL,  $P = .034$ ). LAC<sup>WT</sup>- and LACΔpvl-inoculated rabbits' lung infections did not differ for the percentage of infected lungs, CFU, histological scores, and Hla concentrations.

#### Comparison of LAC<sup>WT</sup> vs LACΔhla Experimental AOM (Table 1)

Among the 19 rabbits infected with LACΔhla, 7 were killed on D3, and 12 were observed until D14.

At the early stage (D3), compared to LAC<sup>WT</sup>, mean LACΔhla bacterial counts in lungs were nonsignificantly lower. However, as for LACΔpvl, LACΔhla-infected animals had significantly less severe histological lung lesions than LAC<sup>WT</sup>-infected animals: inflammation ( $P = .02$ ), lung abscesses ( $P = .007$ ), pleural

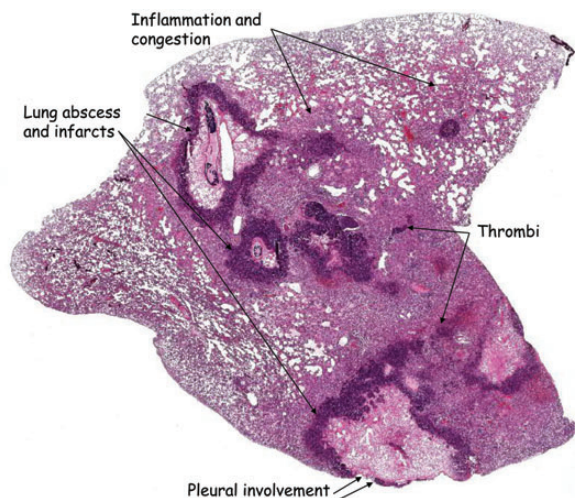


**Figure 1.** Histological scores for microscopic lung lesions. Microscopic lesions were scored (0–4), as described in Methods, for *Staphylococcus aureus* LAC<sup>WT</sup>- ( $\diamond$ ), LAC $\Delta$ *pvl*- ( $\square$ ) and LAC $\Delta$ *hla*-infected ( $\triangle$ ) rabbits killed on D3 or D14, and those that died spontaneously between D3 and D14.

involvement ( $P = .01$ ), megakaryocytes ( $P = .006$ ), bacteria and thrombi ( $P = .007$ ; Figure 1). Notably, PVL was undetectable in the lungs of all LAC $\Delta$ *hla*-infected rabbits. These findings suggest that both PVL and Hla are required for early hematogenous spread to the lungs.

The role of Hla in the systemic severity/hematogenous spread of the infection was also suggested by the observation that, unlike LAC<sup>WT</sup> and LAC $\Delta$ *pvl*, none of the LAC $\Delta$ *hla*-infected rabbits died before D9 (vs 10/19 for LAC<sup>WT</sup>,  $P = .004$ ; and 11/20 for LAC $\Delta$ *pvl*,  $P = .002$ ). Only 2 of the 12 LAC $\Delta$ *hla*-infected

animals died, at late stages (1 each D11 or D14), of severe sepsis with positive spleen and kidney cultures. As for deceased LAC<sup>WT</sup>- and LAC $\Delta$ *pvl*-infected rabbits, those 2 dead LAC $\Delta$ *hla*-infected animals had high bacterial densities in lung with severe histological lesion scores. Kaplan-Meier survival curves for the 3 groups (Figure 4) clearly confirmed the major Hla impact on death (LAC $\Delta$ *hla* vs LAC $\Delta$ *pvl*,  $P = .02$ ; LAC $\Delta$ *hla* vs LAC<sup>WT</sup>,  $P = .03$ ). Median survival was 6 (SD 4) days for LAC<sup>WT</sup>, 6 (SD 3) days for LAC $\Delta$ *pvl*, and could not be computed for LAC $\Delta$ *hla* due to too few deaths in this group.



**Figure 2.** Histological lung section from a  $LAC^{WT}$ -infected rabbit that died on D4. Interstitial congestion and inflammatory infiltrates, abscess, thrombi and infarcts are seen (hematoxylin-and-eosin stain, original magnification  $\times 0.3$ ).

Most D14 survivors had no bacteremia (negative blood, kidney, and spleen cultures). Bone CFU of  $LAC\Delta hla$ -infected rabbits were close to those of D14  $LAC^{WT}$  survivors. However, abscesses, the hallmark of PVL, were less frequent in  $LAC\Delta hla$ -than  $LAC^{WT}$ -infected rabbits (30% vs 88%, Fisher exact test  $P = .01$ ). The percentage of  $LAC\Delta hla$ -induced bone deformation was nonsignificantly lower than that induced by  $LAC^{WT}$  (10% vs 60%). CRP concentrations and anti-PVL antibody titers were intermediate between those of  $LAC^{WT}$ - and  $LAC\Delta pvl$ -infected rabbits (Figure 3).

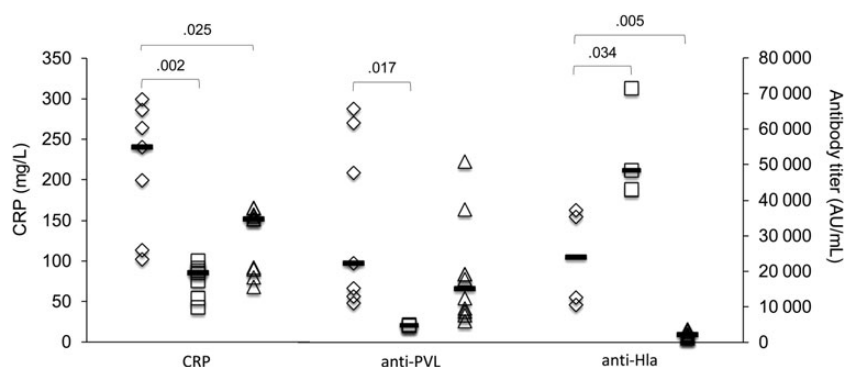
## DISCUSSION

The LAC-rabbit model used in this study closely reproduces the severe AOM seen in children since the onset of the CA-MRSA

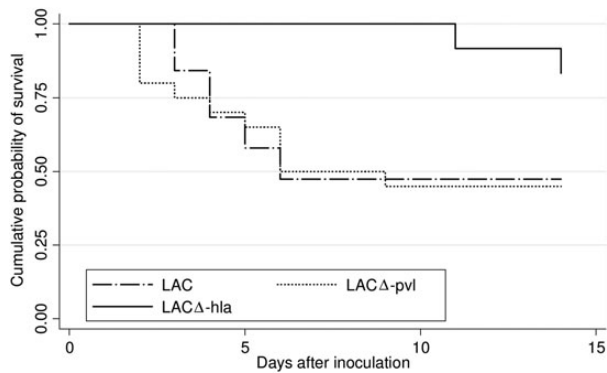
era, with, in many reports [5, 8, 9], substantial numbers of children having disseminated *S. aureus* infection associated with AOM requiring intensive care. Notably, half of the  $LAC^{WT}$ -infected rabbits died between D3 and D9, with severe sepsis, bacteremia, and high bacterial loads in bone and lungs associated with macroscopic and microscopic lung lesions. Although we previously showed that PVL production was associated with local severity (abscess formation, extension to muscle and cortical deformation) of CA-MRSA  $LAC$  experimental AOM [16], the contributions of PVL and Hla to mortality and lung involvement of severe sepsis associated with AOM were not investigated.

Pertinently, the infecting strain's production of both PVL and Hla was required for very early-stage lung involvement/lesions. PVL was detected in the lungs of  $LAC^{WT}$ -infected animals and *pvl* deletion was associated with fewer lung lesions, suggesting a local role for PVL. Unlike PVL, Hla was not detected in lungs on D3. However, when *hla* had been deleted, PVL could no longer be detected, suggesting an Hla role in the early hematogenous spread to lung.

Later on, PVL did not impact mortality or infection dissemination in the rabbits that had succumbed to their infections. In contrast, *hla* deletion dramatically lowered mortality and significantly increased median survival. Notably, high Hla concentrations were detected in  $LAC^{WT}$ -infected animals that died. The strong role of Hla in  $LAC^{WT}$ -associated mortality was described previously in other experimental models [14]. Bubeck et al [15] showed that  $LAC\Delta hla$  was unable to cause lung infection and death of mice given a high intranasal inoculum. Unlike the airborne-LAC model in mice, our rabbit hematogenous infection model results revealed that  $LAC\Delta hla$  was still able to induce AOM complicated by lung involvement. However, in the absence of Hla, the lung involvement was less severe at the early stage (D3), and most rabbits survived, suggesting an important but not exclusive Hla role in the systemic severity associated with experimental AOM. The unusual increase of life-threatening staphylococcal infections observed by pediatricians since the



**Figure 3.** CRP concentrations and anti-PVL and anti-Hla antibody titers in convalescent rabbits sacrificed on D14 and infected with *Staphylococcus aureus*  $LAC^{WT}$  ( $\diamond$ ),  $LAC\Delta pvl$  ( $\square$ ) or  $LAC\Delta hla$  ( $\triangle$ ). Horizontal bars are the medians. Abbreviations: CRP, C-reactive protein; PVL, Panton-Valentine leukocidin.



**Figure 4.** Survival of *Staphylococcus aureus*-infected rabbits according to LAC<sup>WT</sup> (---), LACΔpvl (····), or LACΔhla (—) strain inoculated.

onset of the CA-MRSA era might reflect the exceptionally high production of core-genome–encoded virulence factors (including Hla) by the USA300 CA-MRSA lineage [12, 22], together with PVL synthesis.

In the present model, although the animals survived to the acute septic phase, the subsequent step of the disease was AOM extension accompanied by PVL-dependent extraosseous complications, mimicking those seen in humans [6] and also previously described in the same rabbit model [16]. Pertinently, Hla also contributed, to a lesser extent, to this stage of infection, because LACΔhla-infected animals had fewer abscesses than LAC<sup>WT</sup>-infected rabbits (but more than LACΔpvl-infected rabbits), suggesting a synergistic or redundant effect of these 2 toxins. Their interdependency is also reflected by the unexpected higher anti-Hla antibody titers in LACΔpvl- than LAC<sup>WT</sup>-infected rabbits (albeit tested only in convalescent rabbits sacrificed on D14), suggesting that the deleterious PVL effects on the innate immune system [23, 24] could impair adaptive immunity. Notably, LAC<sup>WT</sup> and LACΔpvl were previously shown to produce similar amounts of Hla [15] (confirmed in this study, not shown) ruling out the possibility that higher anti-Hla titers resulted from higher Hla production.

It is likely that the sequential and synergistic roles of Hla and PVL observed in this rabbit AOM model reflect the complexity of *S. aureus* pathogenesis. Hla could contribute to the systemic manifestations of *Staphylococcus* infection, as assessed by its impact on mortality, whereas PVL, which was previously shown to have a moderate impact on severity during the acute phase of bacteremia [25], could exert a more localized effect, like that observed in late bone abscesses. As suggested by the results of a recent study, the local main effect of PVL could be due to the fact that human serum can mediate protection against PVL epithelium damage by generating PVL-neutralizing antibodies, and by neutralizing the released neutrophil proteases that damage epithelium, via protease inhibitors present in serum [26].

Our results also highlight the need for experimental models that closely reproduce the course of human clinical infections. For example, it was shown, in an experimental postinfluenza model of *S. aureus* superinfection in mice [27], that Hla and protein A were maximally expressed 4 hours after infection, whereas PVL expression peaked 72 hours postinoculation. Because most experimental sepsis models are acute, with mean survival time <4 days [25], the sequential contributions of virulence factors is quite difficult to discern in those models.

However, even though the model used here resembles pediatric hematogenous osteomyelitis in several respects [28], unlike acute osteomyelitis in children, this model requires local inoculation and the use of a sclerosing agent to create small-vessel thrombosis and micronecrosis. Another limitation of this study is that neither LACΔpvl–Δhla double mutants nor animal experiments with chromosomally restored derivatives of the LACΔpvl and LACΔhla strains were used in this AOM model. However, LAC<sup>WT</sup> isogenic deletion strains (LACΔpvl and LACΔhla) were engineered previously by Frank DeLeo's group and validated by numerous studies ([14, 15, 17] and others), thereby diminishing the need for such animal control groups versus ethical considerations.

In conclusion, our experimental results confirm the role of PVL in the local extension of bone infection in those animals who survived long enough for PVL to be expressed in this AOM model. However, they also clearly showed that PVL was not a major factor in sepsis dissemination and death, and that other major virulence factor (eg, Hla) should be considered in the treatment or prevention of these severe infections seen in by pediatricians in the era of USA300 CA-MRSA.

## Notes

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All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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