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Gram Staining of *Coxiella burnetii*

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In routine work examining infected chick embryo yolk sacs with smears stained with Gram-Nicolle stain (Nicolle, Ann. Inst. Pasteur **9**:664, 1895), it was noted that *Coxiella burnetii* reacted occasionally as a gram-variable organism. This observation led to a study of the behavior of the species with variations of the Gram stain.

The primary stains were Hucker's ammonium oxalate-crystal violet, Kopeloff and Beerman's sodium bicarbonate-crystal violet, and Nicolle's phenol-gentian violet; they were flooded on the slides, which had been heat-fixed and cooled. The mordants were either aqueous solutions (containing either 1.0% iodine plus 2.0% potassium iodide, or 0.5% iodine plus 1.0% potassium iodide) or an ethyl alcohol solution made by adding 2 volumes of 2% aqueous solution to 1 or 2 volumes of 95% ethyl alcohol; each was applied for 1 min. The decolorizers were 95% ethyl alcohol for 1 min or ethyl alcohol-acetone (4 volumes of 95% ethyl alcohol plus 1 volume of acetone) for 15 to 18 sec, except as noted; the decolorizers were put in Coplin jars, and the wet slides were immersed in the jar. Decolorizers were replaced after every three slides. The counterstains were 0.2% aqueous safranine for 30 sec or 1% aqueous Bismarck Brown for 1 min. Between steps the slides were washed in tap water. Mordants and counterstains were applied to wet slides by rinsing and flooding.

The aerobic bacterial controls, *Bacillus subtilis*, *Staphylococcus aureus*, a *Corynebacterium* sp., *Streptococcus pyogenes*, *Neisseria catarrhalis*, *N. sicca*, *Escherichia coli*, *Haemophilus influenzae*, *Salmonella typhimurium*, and *Pasteurella multocida*, were cultivated on blood-agar slants and incubated at 37 C for 20 hr; *P. tularensis* and *Brucella melitensis* were cultivated on glucose-cysteine-blood-agar and tryptose-agar, respectively, at 37 C for 20 hr; a *Saccharomyces* sp. and *Nocardia asteroides* were grown in Sabouraud medium at room temperature; *Mycobacterium marinum* was grown on Lowenstein-Jensen medium at 33 C for 7 days. The anaerobic bacterial controls, *Clostridium botulinum* and a *Bacteroides* sp., were cultivated in thioglycolate broth at 37 C for 20 hr. *Rickettsia prowazekii*, *R. mooseri*, *R. rickettsii*, *R. akari*, and *R. tsutsugamushi* were grown by use of usual techniques in yolk sacs of chick embryos at 35 C, and psittacosis organisms were grown in yolk sacs in chick embryos at 37 C.

Light suspensions in 0.85% saline were prepared from the bacterial cultures, and one small

TABLE 1. *Staining reaction of Coxiella burnetii to four variations* of the Gram stain, with aqueous iodine or ethyl alcohol-iodine as mordants*

Organism	Aqueous iodine				Ethyl alcohol iodine			
	A	B	C	D	A	B	C	D
C. burnetii	−†	±	−	±	±	+	+	+
Rickettsiae	−	−	−	−	−	−	−	−
Gram-positive bacteria	+	+	+	+	+	+	+	+
Gram-negative bacteria	−	−	−	−	−‡	−	−	−

* (A) Gram-Hucker according to Bartholomew (Stain Technol. **37**:139, 1962). The aqueous mordant contained 1% iodine. The decolorizer was 95% ethyl alcohol. (B) Gram-Nicolle with phenol-violet for 1 min. The aqueous mordant contained 0.5% iodine; the decolorizer was ethyl alcohol-acetone. (C) Gram-Kopeloff and Beerman according to Conn et al. (p. 16–17, *In* Society of American Bacteriologists, *Manual of Microbiological Methods*, McGraw-Hill Book Co., Inc., New York, 1957), but destaining as in Gram-Nicolle. (D) A modification of Gram-Kopeloff and Beerman in which the primary stain, a mixture of 1% aqueous crystal violet plus the same volume of aqueous 5% sodium bicarbonate (mixture prepared fresh, not suitable after 1 hr), was steamed for 1 min. The aqueous mordant contained 1% iodine; the decolorizer was ethyl alcohol-acetone for 18 sec.

† Results are interpreted as follows: −, gram-negative; ±, gram-variable; +, gram-positive.

‡ *Neisseria catarrhalis* reacted as gram-variable.

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loop of the suspensions was smeared; the dilution was adjusted to avoid overlap of the bacterial cells in the smear. Very thin smears were prepared directly from infected yolk sacs and from ether-extracted antigen of *C. burnetii* (aqueous and washed sediment of Method 1, Topping and Shepard, Public Health Rept. U.S. **61**:701, 1946), as well as directly from infected yolk sacs of other rickettsiae and psittacosis organisms. Every slide was prepared with smears of three gram-positive bacteria, three gram-negative bacteria, one *C. burnetii*, and one rickettsia, alternated in sequence.

Four variations of the Gram stain were tested, as described in Table 1, with either aqueous iodine or ethyl alcohol-iodine solution as a mordant. When aqueous iodine was used, *C. burnetii* occasionally gave a gram-variable reaction with techniques B and D, and a gram-negative reaction with techniques A and C. When ethyl alcohol-iodine was used, *C. burnetii* gave a gram-variable reaction with technique A, and a gram-positive reaction with techniques B and C. Clear-cut results were obtained by use of technique D with ethyl alcohol-iodine: *C. burnetii* was strongly gram-positive, the gram-positive bacterial controls were gram-positive, and the gram-negative bacterial controls, as well as the other rickettsiae and psittacosis organisms, were gram-negative. *C. burnetii* directly from infected yolk sacs and from ether-extracted preparations showed the same staining properties with the Gram stain. The two counterstains gave equivalent Gram reactions.

C. burnetii is considerably different from the other rickettsiae, especially in its great resistance to heat, drying, and antiseptics. Philip (Public Health Rept. U.S. **63**:58, 1948) suggested the name Coxiella as a subgenus of rickettsiae for the organism because it had "certain striking characters." The present findings indicate an even more profound distinction from the other rickettsiae, since, in the Gram-Nicolle technique, *C. burnetii* behaved occasionally as a gram-variable, and it was strongly gram-positive in a modified procedure that stained the bacterial and rickettsial controls with the prescribed results. Earlier antigenic findings indicated another affinity with the gram-positive bacteria. When shaken with ether, gram-negative bacteria and the other rickettsiae released a soluble antigen, whereas gram-positive bacteria and *C. burnetii* did not (Shepard, Public Health Rept. U.S. **61**:54, 1946). A more direct application of the present finding would lie in the possibility of rapid differentiation of *C. burnetii* from other rickettsiae in new isolates.