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Methods for Enhanced Culture Recovery of *Francisella tularensis*

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***Francisella tularensis* is found in a wide variety of hosts and extrahost environments, making culture recovery a diagnostic challenge. Here we demonstrate improved recovery times and good sensitivity (90%) when cultures were inoculated on the site of an investigation using fresh tissues. For contaminated specimens, antibiotic supplementation of enriched cysteine heart agar blood culture medium improved recovery of *F. tularensis* by 81.1%. For transport of tissues, immediate freezing yielded culture recovery rates as high as 94%.**

Francisella tularensis, the etiologic agent of the zoonotic disease tularemia, is found throughout the Northern Hemisphere (Holarctic region) (3, 6). Natural infections with *F. tularensis* have been found in a wide range of vertebrates (~150 species) and invertebrates (~100 species), as well as contaminated soil, water, and vegetation, showing that *F. tularensis* has the ability to spread and survive in widely differing natural environments (7, 10). In nature, the true reservoirs of the bacterium are unknown, although hosts such as ticks, rodents, and protozoa have been suggested (1, 7, 10). The ecology of tularemia is also poorly understood due in large part to the broad distribution of *F. tularensis* in nature.

The prevalence and distribution of *F. tularensis* in nature has recently become of more general interest due to heightened concerns that *F. tularensis* might be used as an agent of bioterrorism (5). Identification of *F. tularensis* by culture presents problems because the organism is highly infectious, fastidious, and grows slowly in vitro (4, 12). *F. tularensis* is also extremely difficult to recover from field specimens, with recovery rates from carcasses at only 30% (9). Nevertheless, the availability of such cultures is essential for detailed molecular epidemiology studies and for our understanding of how biotype and strain variations can impact the natural cycles of *F. tularensis*. Further, in the case of an intentional release of *F. tularensis*, the earliest agent recovery will expedite its characterization and control.

Here, we evaluated several culture and transport systems for their use in enhancing recovery of *F. tularensis* cultures. Comparisons were performed using samples from a tularemia outbreak in prairie dogs (*Cynomys ludovicianus*) (2).

MATERIALS AND METHODS

Outbreak specimens. For comparative evaluations, prairie dogs were divided into group A (46 dead animals) and group B (20 live animals, euthanized). All group A and B animals were previously tested and classified as *F. tularensis* subsp. *holarctica* (type B) positive (11).

On-site specimen processing. All group A and B prairie dogs were necropsied on-site by using a mobile field station. The field station, a 6- by 10-ft closed trailer with fold-out workbenches, contained personal protective equipment, biohazard containment supplies, surface sterilization solvents, sterile necropsy equipment, tissue collection vials, and culture media. Prairie dogs were surface sterilized with ethyl alcohol and necropsied, and tissues were surgically removed. Personal protection included single-use disposable closed-front gowns, N95 masks, glasses, and double gloves.

Transport systems. Within 15 min of necropsy, spleen and liver tissues were frozen or transferred to Cary-Blair transport medium; ~10 g was placed in cryovials and frozen on dry ice, and ~2 g was placed in Cary-Blair transport medium and held at 4°C until transport to the laboratory (~72 h). On arrival at the laboratory, frozen tissues were placed at −20°C and Cary-Blair samples were placed at 4°C. For evaluations, tissues were cultured 3 weeks later.

Culture recovery of *F. tularensis*. Within 15 min of necropsy, spleen and liver tissues were punctured 10 to 20 times with a sterile wooden stick. Tissue adhering to the stick was transferred to cysteine heart agar with chocolatized 9% sheep blood (CHAB), and then a 1-μl sterile loop was used to streak the plate for colony isolation. Plates were sealed immediately with parafilm and transported in ice coolers (~15 to 20°C) until arrival at the Centers for Disease Control and Prevention laboratory, Fort Collins, Colo. (~72 h later). All plates were then transferred to a biosafety level 3 laboratory, where they were incubated at 37°C for 5 more days and checked daily for characteristic *F. tularensis* growth. All recovered isolates were confirmed as *F. tularensis* (11).

CHAB supplemented with 7.5 mg of colistin, 2.5 mg of amphotericin, 0.5 mg of lincomycin, 4 mg of trimethoprim, and 10 mg of ampicillin per liter (CHAB-A) was also utilized. CHAB-A culture plates were inoculated in the laboratory and incubated at 37°C for 7 days.

RESULTS

Rapid recovery of *F. tularensis* cultures. To see if recovery times for *F. tularensis* could be improved, necropsy specimens were collected from all group A and B animals and immediately cultured on the site of an investigation. In order to minimize airborne contamination, the mobile field station was moved into the animal facility and away from direct air-conditioning flow. Despite suboptimal growth temperatures (~15 to 20°C) during transport (72 h), this approach yielded 5 cultures from group A animals and 13 cultures from group B animals on arrival at the laboratory. Upon 24 h incubation at 37°C, an additional three cultures from group A and three cultures from group B animals were obtained. After 4 more days of incubation, the last cultures were obtained: one culture from group A and two cultures from group B animals.

For live animals (group B), the sensitivity of on-site culture inoculation was 90% (Table 1), demonstrating the utility of this technique when testing fresh tissues. In comparison, the sensitivity for carcass tissues (group A) was only 19.6% (Table 1), with a surprisingly low recovery rate of 17% from animals dead

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TABLE 1. Comparison of *F. tularensis* recovery rates for on-site culture and antibiotic-supplemented CHAB (CHAB-A)

Prairie dogs (*n*)	Culture recovery (%)[a] with:	
	CHAB (on-site)	CHAB-A
Group A, live animals (20)	18 (90)	ND
Group B, carcasses (46)	9 (19.6)	30 (81.1)[b]

[a] The difference in on-site recovery rates for group A versus group B and the difference in recovery rates for carcass tissues cultured on CHAB versus CHAB-A were statistically significant ($P \leq 0.05$, Fisher's exact test). ND, not determined.
[b] Thirty-seven CHAB culture-negative prairie dogs were tested.

TABLE 2. Comparison of transport methods for culture recovery of *F. tularensis* from live and dead animals[a]

Prairie dogs (*n*)[b]	Culture recovery (%) with:	
	Cary-Blair media	Frozen tissue
Carcasses (9)	1 (11.1)	8 (88.8)
Live animals (17)	15 (88.2)	16 (94.1)

[a] No statistically significant difference was observed when transport systems were compared for recovery of *F. tularensis* from live animals ($P \geq 0.05$, McNemar's test). The difference in transport systems for dead animals was statistically significant ($P \leq 0.05$, McNemar's test).
[b] Twenty-six animals from which *F. tularensis* was cultured by on-site inoculation of CHAB plates.

less than 24 h (data not shown). The majority of cultures initiated from carcass tissues ($n = 37$) yielded only *Pseudomonas*, *Staphylococcus*, or *Proteus* species.

Antibiotic-supplemented media required for culture recovery from contaminated specimens. To determine if cultivable *F. tularensis* was present in culture-negative carcass tissues, CHAB medium was supplemented with a combination of antibiotics (CHAB-A) previously shown to preserve the viability of *F. tularensis* (8). With CHAB-A, 30 *F. tularensis* isolates were cultured from the 37 CHAB-negative carcasses, improving the recovery rate by 81.1% (Table 1). When side-by-side comparisons were performed with CHAB versus CHAB-A, we found that *F. tularensis* growth was dramatically inhibited by the presence of contaminating bacteria in carcass tissues (e.g., Fig. 1). Thus, isolation of *F. tularensis* from contaminated specimens requires the utilization of antibiotics to suppress growth of inhibitory bacterial species.

Comparison of transport systems. Two transport systems were also evaluated for their utility in the recovery of live organisms from tissues that cannot be immediately cultured (Table 2). For recovery of *F. tularensis* from carcasses, a statistically significant difference between transport systems was observed. The recovery rate from frozen tissue was 88.8%, while the recovery rate from Cary-Blair medium was only 11.1% ($P \leq 0.05$, McNemar's test) (Table 2). In the majority of cases, cultures initiated from tissues transported in Cary-Blair medium were highly contaminated with *Pseudomonas*, *Staphylococcus*, and *Proteus* species.

There was no statistically significant difference observed when transport systems were compared for recovery of *F. tularensis* from live animals. The recovery rate from frozen tissue was 94.4%, while the recovery rate from Cary-Blair medium was 83.3% ($P \geq 0.05$, McNemar's test) (Table 2).

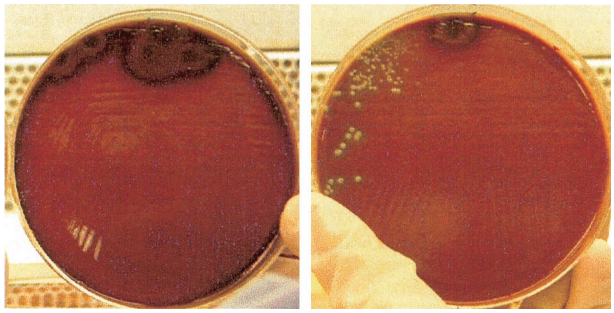

FIG. 1. Comparison of carcass tissues plated on CHAB (left panel) and on CHAB-A (right panel).

DISCUSSION

Due in large part to the hazards associated with culture of *F. tularensis* and the low funding priority of tularemia research in recent decades, little effort has focused on the evaluation of different systems for the efficient recovery of live organisms from field specimens. In the present study, we identified and evaluated culture and transport systems that demonstrate good sensitivity, improved culture recovery times, and recovery rates as high as 84.8% from infected carcasses, specimens that are usually highly contaminated and pose a significant challenge for isolating *F. tularensis*. Although these methods were evaluated on specimens infected with *F. tularensis* subsp. *holarctica* (type B), we expect the methods to be fully applicable to *F. tularensis* subsp. *tularensis* (type A), as these two subspecies have highly similar culture characteristics and growth requirements.

Culture is often not the preferred diagnostic method for *F. tularensis* when a rapid result is required, as it is a fastidious, slow-growing organism. However, by inoculating CHAB plates on the site of an investigation, we were able to shorten culture recovery times that would have otherwise been delayed by shipping (e.g., 3 to 6 days saved by eliminating normal transport times). This technique also showed very good sensitivity (90% recovery rate) when cultures were initiated from tissues of live *F. tularensis*-infected animals. Most likely, this success was due to the freshness of the specimens used for culture; as soon as the animals were euthanized, tissues were excised and immediately cultured, thus minimizing environmental contamination.

Recovery of live *F. tularensis* from field specimens has historically proven to be a significant diagnostic challenge. Here, we showed in the case of carcasses that recovery of *F. tularensis* is negatively affected by the growth dynamics of competing bacteria and that this effect occurs within 24 h of death. Antibiotic supplementation of CHAB media controlled the growth of contaminating bacteria and significantly improved the ability to recover *F. tularensis* ($P \leq 0.05$), allowing for an increase in sensitivity of 81.1%.

Whether other bacteria deplete nutrients required for growth of *F. tularensis* and/or produce bacteriocins affecting *F. tularensis* growth requires further study. Nonetheless, these findings have important implications when attempting to isolate *F. tularensis* from a variety of samples that contain other bacterial species. To date, CHAB-A media has proved useful

for culture of *F. tularensis* from carcasses and urine. Future evaluations of this media will also be important using water, soil, and grass or hay.

In this study, we also compared culture recovery from infected tissues after use of different transport systems. Freezing of tissues from carcasses yielded isolates in 75% of cases and afforded significantly greater transport recovery than did Carey-Blair medium ($P \leq 0.05$). This difference is likely due to postmortem growth dynamics of competing bacteria that continue to evolve in Carey-Blair medium. We conclude that freezing of tissues is more likely to preserve predeath bacterial distributions and enable improved recovery *of F. tularensis*. Alternatively, Cary-Blair medium supplemented with the antibiotics described here may also be used for transport but will require future evaluation.

In summary, utilization of the culture and transport systems described and evaluated in this study should prove useful for testing a wide variety of samples, thus providing important new insights into the ecology, epidemiology, and transmission cycles of *F. tularensis* and improving our bioterrorism response capabilities.

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REFERENCES

1. **Abd, H., T. Johansson, I. Golovliov, G. Sandstrom, and M. Forsman.** 2003. Survival and growth of *Francisella tularensis* in *Acanthamoeba castellanii.* Appl. Environ. Microbiol. **69:**600–606.
2. **Centers for Disease Control and Prevention.** 2002. Public health dispatch: outbreak of tularemia among commercially distributed prairie dogs, 2002. Morb. Mortal. Wkly. Rep. **51:**688–689.
3. **Centers for Disease Control and Prevention.** 2002. Tularemia-United States, 1990–2000. Morb. Mortal. Wkly. Rep. **51:**182–184.
4. **Chu, M. C., and R. Weyant.** 2003. *Francisella* and *Brucella*, p. 789–797. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. A. Pfaller, and R. H. Yolken (ed.), Manual of clinical microbiology, 8th ed. American Society for Microbiology, Washington, D.C.
5. **Dennis, D. T., T. V. Inglesby, D. A. Henderson, J. G. Barlett, M. S. Ascher, E. Eitzen, A. D. Fine, A. M. Friedlander, J. Hauer, M. Layton, S. R. Lillibridge, J. E. McDade, M. T. Osterholm, T. O'Toole, G. Parker, T. M. Perl, P. K. Russell, and K. Tonat.** 2001. Tularemia as a biological weapon: medical and public health management. JAMA **285:**2763–2773.
6. **Ellis, J., P. C. F. Oyston, M. Green, and R. W. Titball.** 2002. Tularemia. J. Clin. Microbiol. **15:**631–646.
7. **Hopla, C. E., and A. K. Hopla.** 1994. Tularemia, p. 113–126. *In* G. W. Beran and J. H. Steele (ed.), Handbook of zoonoses, 2nd ed. CRC Press, Inc., Boca Raton, Fla.
8. **Johansson, A., L. Berglund, U. Eriksson, I. Göransson, R. Wollin, M. Forsman, A. Tärnvik, and A. Sjöstedt.** 2000. Comparative analysis of PCR versus culture for diagnosis of ulceroglandular tularemia. J. Clin. Microbiol. **38:**22–26.
9. **Mörner, T., G. Sandstrom, R. Mattsson, and P. O. Nilsson.** 1988. Infections with *Francisella tularensis* biovar *palaearctica* in hares (*Lepus timidus*, *Lepus europaeus*) from Sweden. J. Wildl. Dis. **24:**422–433.
10. **Mörner, T.** 1992. The ecology of tularemia. Rev. Sci. Tech. Off. Int. Epizoot. **11:**1123–1130.
11. **Petersen, J. M., M. E. Schriefer, L. G. Carter, Y. Zhou, T. Sealy, D. Bawiec, B. Yockey, S. Urich, N. S. Zeidner, S. Avashia, J. Kool, J. Buck, C. Lindley, L. Celeda, J. A. Monteneiri, K. L. Gage, and M. C. Chu.** 2003. Laboratory analysis of a tularemia outbreak in wild trapped, commercially traded prairie dogs: isolation of *Francisella tularensis* from seropositive animals. Emerg. Infect. Dis. **10:**419–425.
12. **Sjöstedt, A.** Family XVII. *FRANCISELLACEAE*, Genus I. *Francisella. In* D. J. Brenner (ed.), Bergey's manual of systemic bacteriology, in press. Springer-Verlag, New York, N.Y.