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Selective isolation of *Yersinia pestis* from plague-infected fleas

Derek S. Sarovich[1], **Rebecca E. Colman**[1], **Erin P. Price**[1], **Wai Kwan Chung**[1], **Judy Lee**[1], **James M. Schupp**[1], **James Alexander**[2], **Paul Keim**[1], and **David M. Wagner.**[1,1*]

[1]Center for Microbial Genetics and Genomics, Northern Arizona University, Flagstaff, Arizona, USA

[2]Texas Department of State Health Services, Canyon, Texas, USA

Abstract

We evaluated *Yersinia* CIN agar for the isolation of *Yersinia pestis* from infected fleas. CIN media is effective for the differentiation of *Y. pestis* from flea commensal flora and is sufficiently inhibitory to other bacteria that typically outcompete *Y. pestis* after 48 hours of growth using less selective media.

Keywords

plague; *Yersinia pestis*; isolation; CIN agar; selective

Yersinia pestis, the causative agent of plague, regularly causes die-offs of prairie dogs (*Cynomys* spp.) and other rodent species in the western United States. *Y. pestis* is transmitted between rodent hosts by infected fleas. Following death of the rodent hosts, plague-infected fleas can be readily collected from rodent burrows and can serve as an important source of *Y. pestis* genomic DNA (gDNA) (Allender et al., 2004) and isolates (Girard et al., 2004), as access to infected or dead animals is not always possible or practical. The presence of *Y. pestis* in fleas can be confirmed via PCR using *Y. pestis*-specific targets and extracted gDNA from infected fleas (Allender, Easterday, Van Ert, Wagner and Keim, 2004, Girard, Wagner, Vogler, Keys, Allender, Drickamer and Keim, 2004, Stevenson et al., 2003). However, to perform more detailed analysis of *Y. pestis*, direct culturing and isolation is often desirable.

Plague isolates are commonly obtained from infected fleas via the inoculation of triturated flea material into laboratory mice (Barnes, 1982, Engelthaler et al., 1999, Hinnebusch et al., 1998, Poland, 1979, Politzer, 1954, Quan et al., 1981). Although this method has been the gold standard for plague detection, it is laborious, costly, and requires several days before diagnosis is obtained (Engelthaler, Gage, Montenieri, Chu and Carter, 1999). In addition, surviving mice are not routinely examined, which can be problematic as these mice may be infected, yet not succumb within the timeframe of the study (Engelthaler, Gage, Montenieri, Chu and Carter, 1999). Lastly, mouse inoculation involves specialized animal handling facilities and the sacrifice of mice to obtain live culturea. As such, direct culture of *Y. pestis* from the fleas may be a more desirable method than mouse inoculation, but has proven problematic due to the native flora of fleas, which commonly outcompetes the slower-growing *Y. pestis* (Baltazard et al., 1956, Engelthaler and Gage, 2000, Engelthaler et al., 2000, Hinnebusch, Gage and Schwan,

*Corresponding author: David M. Wagner, Center for Microbial Genetics and Genomics, Northern Arizona University, Flagstaff, Arizona, United States 86011-4073, dave.wagner@nau.edu, Ph: (928) 523-0686, Fax: (928) 523-0639.

1998, Kartman and Prince, 1956, McDonough et al., 1993, Quan et al., 1960, Thomas et al., 1989).

Cefsulodin, irgasan, novobiocin (CIN) agar (Hardy Diagnostics, Santa Maria, CA) was developed for the isolation of *Yersinia enterocolitica* (Schiemann, 1979) and has been previously used for the testing of clinical specimens for presence of *Y. pestis* (Rasoamanana et al., 1996). However, direct isolation of *Y. pestis* from infected fleas on CIN agar has never been reported. Here, we describe a method for the direct isolation of *Y. pestis* from whole fleas using CIN agar.

In June 2009 we collected fleas (*Oropsylla* spp.) from a black-tailed prairie dog (*Cynomys ludovicianus*) population in Hansford County, Texas that exhibited signs of a recent die-off. A total of 569 fleas were collected from 14 different burrows using previously described techniques (Girard, Wagner, Vogler, Keys, Allender, Drickamer and Keim, 2004). A subset of 125 fleas from nine burrows were used for culturing. These fleas were pooled by burrow, with a maximum of ten fleas per tube, and 150uL of brain heart infusion broth (BD Diagnostics, Sparks, MD) supplemented with 10% glycerol was added to the tubes. Fleas were ground using a sterile pellet pestle (Kimble Chase, Vineland, NJ) and a small amount of fine sterile beach sand (EMD Chemicals INC, Gibbstown, New Jersey). Once the fleas were adequately ground (indicated by rupture of the mid-guts), the mixture was transferred to a single CIN agar plate and incubated at 28°C for 48 hours. Following incubation, *Y. pestis* colonies appeared similar to previously described *Y. enterocolitica* colonial growth (Schiemann, 1979). Specifically, colonies were 1-2 mm in size, dark red, and raised with fried egg morphology (Figure 1). Although some non-*Y. pestis* growth was observed after 48 hours, CIN agar was sufficient at inhibiting competitor growth and allowed differentiation of *Y. pestis* from commensal species. Single suspect *Y. pestis* colonies were subcultured onto sheep blood agar (SBA; Hardy Diagnostics) and gDNA was extracted using heat lysis (Keim et al., 2000). *Y. pestis* identity was confirmed using a real-time PCR-based assay targeting the plasmid borne *pla* gene (Hinnebusch and Schwan, 1993, Stevenson, Bai, Kosoy, Montenieri, Lowell, Chu and Gage, 2003); this same assay was used to confirm the presence of *Y. pestis* DNA in DNA extracts from the remaining 444 fleas that were not used for culturing.

Previous studies have highlighted the difficulties of direct isolation of *Y. pestis* from field-collected fleas as commensal flora quickly overgrows *Y. pestis* on nonselective media (Baltazard, Davis, Devignat, Girard, Gohar, Kartman, Meyer, Parker, Pollitzer, Prince, Quan and Wagle, 1956, Engelthaler and Gage, 2000, Engelthaler, Hinnebusch, Rittner and Gage, 2000, Hinnebusch, Gage and Schwan, 1998, Kartman and Prince, 1956, McDonough, Barnes, Quan, Montenieri and Falkow, 1993, Quan, Kartman, Prince and Miles, 1960, Thomas, McDonough and Schwan, 1989). In addition, we have previously attempted isolation of *Y. pestis* from fleas using SBA (data not shown). However, due to the relatively slow growth rate of *Y. pestis* compared with many of the commensal bacterial species present in and on fleas, and an inability to morphologically distinguish *Y. pestis* from other bacteria on SBA, *Y. pestis* could not be easily isolated using this agar.

Unlike SBA, CIN media is inhibitory to many bacteria such as *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Shigella sonnei* and *Streptococcus* spp., due to the presence of the selective agents sodium deoxycholate, cefsulodin, irgasan and novobiocin (Schiemann, 1979). We have found that even when using this highly selective medium, contaminants are evident at 48 hours and begin to overgrow *Y. pestis* colonies after 72 hours of growth. Therefore, we recommend that colonial interpretation for *Y. pestis*-positive growth on CIN agar and subculture onto SBA be carried out at 48h to maximize species specificity.

One shortcoming of CIN agar is the purported reduction of *Y. pestis* recoverability compared with other selective media. There have been several previous attempts to create media for the enrichment of *Y. pestis* (Ber et al., 2003, Ber et al., 2003, Drennan and Teague, 1917, Markenson and Ben-Efraim, 1963, Meyer and Batchelder, 1926, Morris, 1958) and, although most offer improved recoverability, these media are less inhibitory to non-*pestis* contaminants, making them unsuitable for the direct isolation of *Y. pestis* from fleas. CIN agar differs from these enrichment media in that it is highly selective, but this comes with the tradeoff of reduced enrichment of this fastidious organism. Despite this drawback, we obtained *Y. pestis* isolates from fleas collected from three of nine burrows. Multiple *Y. pestis*-infected fleas were identified from these three burrows using PCR (data not shown). In addition, at least one *Y. pestis*-infected flea was identified from each of the six burrows from which cultures were not obtained. The negative culture results for these six burrows compared to PCR is likely due to the smaller number of fleas used for culture (N=125) versus PCR (N=444), and the low number of overall *Y. pestis*-infected fleas (46/444 fleas tested with PCR).

In conclusion, we have shown that CIN agar is highly selective for *Y. pestis* and sufficiently inhibits growth of common flea commensal species to allow the isolation of *Y. pestis*. We recommend the use of this medium for the selective isolation of *Y. pestis* from plague-infected fleas.

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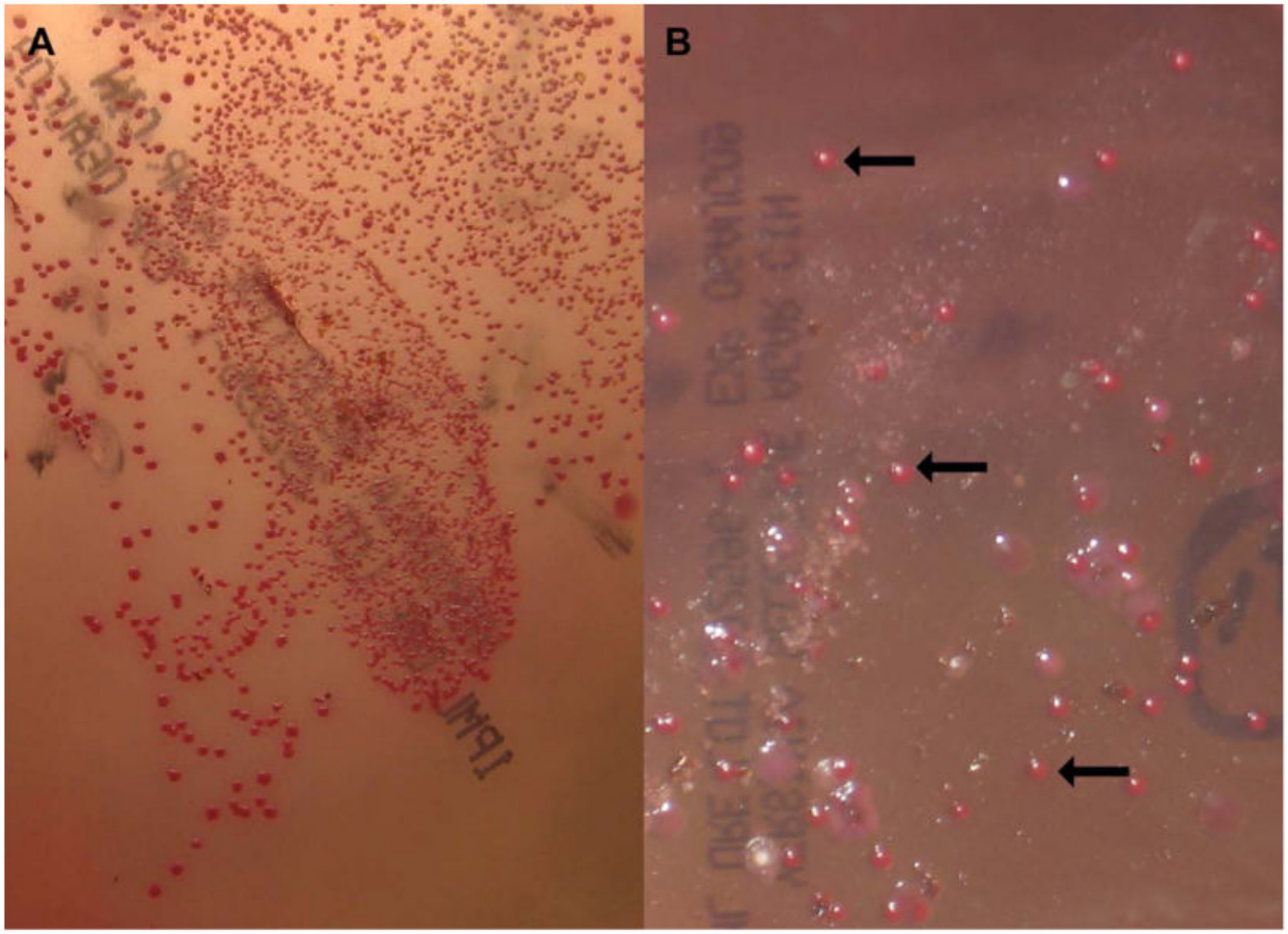

Figure 1. *Yersinia pestis* colonies isolated from whole fleas using cefsulodin, irgasan, novobiocin (CIN) media

Triturated flea material was incubated on CIN media for 48 hours (A) or 72 hours (B) at 28° C before presumptive identification. *Y. pestis* colonies were readily discriminated by colonial morphology of 1-2mm in diameter and bright red with raised fried egg morphology. Panel A shows predominately *Y. pestis* growth whereas panel B shows *Y. pestis* and contaminant growth after 72 hours. Arrows in panel B point to *Y. pestis* colonies.