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ORIGINAL ARTICLE

Viability of *Yersinia pestis* subcultures in agar stabs

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Significance and Impact of the Study: We report the ability of *Yersinia pestis* to survive up to 47 years in agar stabs, in rubber-stoppered tubes, under refrigeration (+4 to +10°C), although overall subculture recovery rates were poor and inversely related to the length of time stored. Genetic characterization of virulence gene presence among these subcultures was suggestive of significant variation in the genomic stability of *Y. pestis* subcultures stored under these conditions. This variation, together with all of the inherent temporal, geographic and other genetic variation represented by all of the recoverable strains in the historical 'Collection of *Yersinia pestis*' (Fiocruz-CYP) maintained by the SRP of FIOCRUZ-PE in Brazil was preserved in new frozen culture stocks stored at −70°C as a result of this study. These frozen culture stocks represent a valuable resource for future comparative studies of *Y. pestis*.

Keywords
collection, genomic stability, plague, viability, *Yersinia pestis*.

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Abstract

Since its identification as the causative agent of plague in 1894, thousands of *Yersinia pestis* strains have been isolated and stored. Here, we report the ability of *Y. pestis* to survive up to 47 years in agar stabs, in rubber-stoppered tubes, under refrigeration (+4 to +10°C), although overall subculture recovery rates were poor and inversely related to the length of time stored. Genetic characterization of virulence gene presence among these subcultures was suggestive of significant variation in the genomic stability of *Y. pestis* subcultures stored under these conditions. Specifically, we found variation in the presence of plasmid and chromosomal virulence markers (genes *pla*, *lcrV*, *caf1* and *irp2*) among multiple subcultures of *Y. pestis* strains in the 'Collection of *Yersinia pestis*' (Fiocruz-CYP) maintained by the SRP of FIOCRUZ-PE in Brazil. This variation, together with all of the inherent temporal, geographic and other genetic variation represented by all of the recoverable strains in this historical collection was preserved in new frozen culture stocks stored at −70°C as a result of this study. These frozen culture stocks represent a valuable resource for future comparative studies of *Y. pestis*.

Introduction

Yersinia pestis, a Gram-negative bacterium in the Enterobacteriaceae family, is the causative agent of plague, a flea-transmitted rodent zoonosis that can affect humans and other mammals. It has been responsible for three recognized pandemics throughout history and is currently maintained in established enzootic foci in Africa, Asia and the Americas (Perry and Fetherston 1997). Available evidence suggests that it first arrived in Brazil through the port of Santos, SP, in 1899, during the third pandemic. Over the next several years, *Y. pestis* spread inland and established several natural foci among rodent populations, mainly in the Northeast region (Pollitzer 1954; Pollitzer and Meyer 1965; Baltazard 2004). Subsequent plague surveillance and control activities in these established foci from 1966 to 1997 led to the isolation of hundreds of *Y. pestis* strains from rodents, fleas and humans in these areas (Almeida *et al.* 1985, 1989; Baltazard 2004). The *Y. pestis* strain collection resulting from these activities is a unique and irreplaceable resource, containing all of the available temporal, geographic and genetic diversity of

Y. pestis strains from Brazil, and is of incalculable value for future comparative studies of Brazilian plague (Tavares *et al.* 2012). Preservation of this collection is thus of the highest importance. In recognition of its importance, this culture collection was institutionalized in 2007 and named 'Collection of *Yersinia pestis*' with the acronym Fiocruz-CYP and is currently maintained by the National Plague Reference Service (SRP) of FIOCRUZ-PE (Rocha *et al.* 2009a,b).

Various subsets of strains from the Fiocruz-CYP have been studied using a variety of different approaches (e.g. plasmid content, outer membrane protein profile, RAPD, ribotyping, MLVA, PFGE and CRISPR typing), all aimed at enhancing the understanding of the spread and population dynamics of *Y. pestis* in Brazil (Mello 1970; Burrows and Gillett 1971; Karimi *et al.* 1974; Hudson *et al.* 1976; Abath *et al.* 1989; Leal *et al.* 1997; Cavalcanti *et al.* 2002; Oliveira *et al.* 2012; Barros *et al.* 2013, 2014). In the course of these various studies, most of the original strain cultures were subcultured and the new subculture tubes kept together with the older subcultures, resulting in multiple stored subcultures of each strain created at different times. In the present study, we evaluated the impact of prolonged storage on the viability and genomic stability of these *Y. pestis* subcultures.

Results and discussion

In this study, we assessed the viability and genomic stability of *Y. pestis* subcultures stored in agar stabs, in rubber-stoppered tubes, under refrigeration (+4 to +10°C). Overall, recovery rates were relatively poor. Of the 2428 subculture tubes from 907 strains (1–7 subcultures/strain) from which growth was attempted, 824 (824/2428 = 33·9%) pure subcultures from just 374 (374/907 = 41·2%) strains (1–6 subcultures/strain) were recovered. The rate of recovery was inversely related to the length of time stored, with only 11·2% of the oldest subcultures recovered compared to 87·5% of the most recently stored subcultures. The subculture tubes from which *Y. pestis* was recovered were dated from between 1967 and 2009, indicating that *Y. pestis* remained viable for up to 47 years when stored in agar stabs, in rubber-stoppered tubes, under refrigeration (+4 to +10°C) (Table 1).

Previous reports have indicated the robustness of *Y. pestis* cultures over time. Pollitzer (1954) reported that *Y. pestis* cultures could remain viable for months if kept at temperatures below 20°C, and even for many years if refrigerated. Similarly, Francis (1949) reported both viability and the maintenance of virulence, an indirect measure of genomic stability, following 25 years of storage, without subculturing, of *Y. pestis* cultures.

Table 1 Viability of *Yersinia pestis* subcultures in test tubes

Year subcultures stored	Subculture age (years)	No. of viable subcultures	No. of tested subcultures	Rate of recovery (%)
1967–1971	43–47	54	482	11·2
1972–1976	38–42	138	533	25·9
1977–1981	33–37	182	481	37·8
1982–1986	28–32	64	183	35·0
1987–1991	23–27	186	460	40·4
1992–1996	18–22	26	55	47·3
1997–2001	13–17	100	130	77·0
2002–2006	8–12	48	63	76·2
2007–2009	5–7	21	24	87·5
Without Information	Unknown	5	17	29·4
Total		824	2428	33·9

Although the longevity of many of the examined subcultures was impressive, the overall recovery rate was disappointing, with only 33·9% of the subcultures and 41·2% of the strains from which growth was attempted successfully recovered. This disappointing recovery rate was likely due to the storage of the strains in agar stabs under refrigeration, rather than as frozen stocks in ultra-low storage, or some other more robust storage system (i.e. lyophilization) (Gherna 2010). The low recovery rate was also likely exacerbated by the long storage times of many of the subcultures (Table 1). Other storage methods can reliably maintain viability for much longer periods (Gherna 2010). Alternatively, some of the unrecoverable subcultures could have contained *Y. pestis* cells that had entered a viable but nonculturable (VBNC) state, as has been observed for *Y. pestis* under some conditions (Pawlowski *et al.* 2011). However, further testing would be needed to determine if this was the case here. Regardless, recovery rates of these strains will hopefully be improved in future by the preparation of new frozen culture stocks stored at −70°C for all of the recoverable and characterized strains in this study.

Genomic stability of the recovered subcultures, as measured by the presence of four *Y. pestis* virulence markers, was similarly poor. Specifically, of the 612 recovered subcultures that were assessed by M-PCR for the presence of *pla*, *lcrV*, *caf1* and *irp2*, only 230 (37·6%) of the subcultures were found to possess all four virulence genes. Three or two virulence genes, respectively, were detected in another 220 (35·9%) and 121 (19·8%) of the tested subcultures. Of the remaining tested subcultures, 7 (1·1%) possessed only *lcrV*, 5 (0·8%) possessed only *caf1* and 29 (4·7%) possessed only *irp2*. The *pla* gene was not found alone in any of the tested subcultures. Although the original cultures were not screened for the presence of these four virulence genes, it is likely that the genes were

present when the cultures were first isolated, making the above assessment a reasonable estimate of the retention frequency of these genes in subcultures stored in this fashion. Supporting this assumption is the general lack of genetic diversity among *Y. pestis* strains from Brazil (Cavalcanti *et al.* 2002; Barros *et al.* 2013, 2014), the nearly ubiquitous presence of these genes in virulent *Y. pestis* strains (Perry and Fetherston 1997), and the isolation of these strains during activities associated with surveillance of active disease, suggesting that all of these strains were originally virulent. It should be noted, however, that possession of all four virulence genes is not necessarily required for virulence (Leal-Balbino *et al.* 2004). Thus, it is possible, although unlikely, that some of the original strains could have been missing one or more of these genes, making the above assessment an underestimate of the retention frequency of these virulence genes in subcultures stored in this manner. Table 2 shows the virulence marker combinations found among the tested subcultures. New frozen culture stocks in BHI + Glycerol (25%) were prepared for all 612 recovered and characterized subcultures and stored at −70°C.

This less than ideal virulence marker retention rate is likely due to subculturing of the original cultures over the years. Indeed, the negative effects of subculturing on the virulence of *Y. pestis* have long been known, with repeated subculturing even being recommended as a means of producing avirulent strains (Pollitzer 1954). Unfortunately, it was impossible to formulate an exact rate of virulence marker loss as linked to subculture frequency for the subcultures examined in this study, as the exact passage history for each retained subculture proved impossible to determine. These subculture-tracking difficulties were due to differences in subculture handling and documentation procedures that occurred over the years. The Fiocruz-CYP has been a resource for many research and other plague surveillance-related services over the years (Rocha *et al.* 2009b) and, as such, has been accessed numerous times by various professionals for various purposes and at various times since the original cultures were isolated and stored, leading to the confusion in passage histories for the various strain subcultures in the collection.

The genomic stability results observed here were consistent with those observed previously. In a study of 107 Fiocruz-CYP strains completed nearly 20 years ago, a little over half (54%) of the examined strains retained the three typical virulence plasmids, pPst, pYV and pFra, with the remaining strains found to lack one or two of the plasmids (Leal *et al.* 1997). Unfortunately, direct comparisons between these results and the present study were not possible due to the inability to identify which subculture tube for a particular strain was utilized previously and whether that same subculture was recovered here. However, general comparisons could be made. For example, the three virulence plasmids, as indicated by the *pla*, *lcrV* and *caf1* gene markers, were found to be present in 46·2% of the subcultures examined here. This is an apparent decrease in virulence plasmid retention compared to the previous study and could reflect an actual decrease in the 20 years since the previous study, or could simply be a result of the larger number of strains and subcultures examined here. Likewise, there were some differences in the frequencies of subcultures found to lack the different plasmids seen here *vs* those seen previously. In the present study, the frequency of subcultures lacking the *pla* gene (278 subcultures, 45·4%) was higher than the frequency of subcultures lacking the other markers, suggesting higher instability of the pPst plasmid. The frequencies of subcultures lacking the other markers were substantially lower and similar to each other, with 86 (14·1%), 103 (16·8%) and 118 (19·3%) of the subcultures lacking the *caf1* (pFra), *irp2* (chromosomal *Yersinia* HPI) and *lcrV* (pYV) markers respectively (Table 2). In contrast, Leal *et al.* (1997) found that pFra was the most frequently missing plasmid, followed by pPst, and pYV, although, again, the source of these differences was unclear. Several other studies have reported similar changes in the genome, both in the plasmids and on the chromosome, of Brazilian *Y. pestis* strains following

Table 2 Combination of the virulence markers found among the strains

1 Marker		2 Markers		3 Markers		4 Markers	
Markers	No. (%)	Markers	No. (%)	Markers	No. (%)	Markers	No. (%)
pla	0 (–)	*pla/lcrV*	3 (0·5)	*pla/lcrV/caf1*	53 (8·7)	*pla/lcrV/caf1/irp2*	230 (37·6)
lcrV	7 (1·1)	*pla/caf1*	3 (0·5)	*pla/lcrV/irp2*	13 (2·1)		
caf1	5 (0·8)	*pla/irp2*	3 (0·5)	*pla/caf1/irp2*	29 (4·7)		
irp2	29 (4·7)	*lcrV/caf1*	32 (5·2)	*lcrV/caf1/irp2*	125 (20·4)		
		lcrV/irp2	31 (5·0)				
		caf1/irp2	49 (8·0)				
No. = 612	41 (6·7)		121 (19·8)		220 (35·9)		230 (37·6)

prolonged storage and laboratory handling (Almeida *et al.* 1993, 1994; Leal *et al.* 2000; Leal-Balbino *et al.* 2004).

Altogether, the Fiocruz-CYP represents a valuable and irreplaceable resource. Despite the potential drawbacks inherent in the unclear passage histories of the various subcultures and loss of at least some genomic content in many subcultures, this collection represents all of the available temporal, geographic and genetic diversity of *Y. pestis* strains from Brazil. As such, the recovery, characterization and preservation as new frozen culture stocks of 612 representatives of this collection reported here constitutes an important and timely step in the preservation of this resource for future studies.

Material and methods

The isolates of the Fiocruz-CYP were stored in agar stabs, in rubber-stoppered tubes, under refrigeration (+4 to +10°C). Due to the classification of *Y. pestis* as a biological Risk Group 3 and Category A bioterrorism agent, the Fiocruz-CYP cultures are currently kept and handled in a Biosafety Level 3 (BSL-3) laboratory (Rocha *et al.* 2009b). For this study, subcultures were plated on blood agar plates and incubated for 3–5 days at 28°C. Confirmation of the subcultures' purity and identification as *Y. pestis* were determined using a bacteriophage test, as previously described (Karimi 1978). The presence of four virulence markers (*pla*, *lcrV*, *caf1* and *irp2* genes located on the pPst, pYV and pFra plasmids, and in the *Yersinia* high-pathogenicity island (HPI) respectively) was determined for up to three recovered subcultures per strain using a Multiplex-PCR (M-PCR) analysis, as previously described (Leal and Almeida 1999).

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Conflict of Interest

No conflict of interest declared.

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