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Identification of encapsulated and non-encapsulated *Yersinia pestis* by immunofluorescence tests using polyclonal and monoclonal antibodies

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SUMMARY

Rabbit polyclonal hyperimmune antibodies to *Yersinia pestis*, and a mouse monoclonal antibody against the capsular antigen fraction 1 (F1) were compared in immunofluorescence (IF) tests. Fluorescent antibody conjugates were prepared from polyclonal antisera to four F1 positive *Y. pestis* strains; the conjugated antibody to strain A1122 gave the strongest IF staining of F1 positive and F1 negative *Y. pestis* strains. Indirect assays were rejected in favour of direct assays utilizing polyclonal and monoclonal reagents because the increased background staining reduced the effective contrast of bacterial visualisation. Polyclonal conjugates gave fairly homogeneous staining of *Y. pestis* bacterial populations, but in monoclonal assays a skew distribution of fluorescence intensity was observed, the majority of bacteria being poorly stained. The proportion of cells stained well by the monoclonal sufficed for easy identification of *Y. pestis* of the F1 positive phenotype however, and staining was not affected by washing the bacteria or treating them with formaldehyde. *Y. pestis* strains of the F1 positive genotype reacted with the monoclonal if bacteria were grown at 37 °C but not if the growth temperature was reduced to 25 °C thus preventing capsule production. The polyclonal conjugate reacted with bacteria of these strains that had been grown at either temperature. Strains of F1 negative genotype grown at either temperature reacted with the polyclonal conjugate but not with the monoclonal. Cross reactions between the polyclonal reagents and *Y. enterocolitica* biovar 2, serovar O 8 could not be removed by selective absorption; however, the monoclonal antibody gave no cross reaction.

The F1 phenotypic status of bacterial preparations was verified by ELISA measurement of the fraction 1 antigen concentration. Antigen levels for F1 positive and F1 negative phenotypes differed by about three logs for suspensions of *Y. pestis* harvested from solid media.

The polyclonal and monoclonal direct IF tests applied to spleen and blood smears of laboratory mice infected with *Y. pestis* were able to differentiate

between lethal infection with an F1 positive strain carrying all four classical virulence determinants, an F1 positive vaccine strain, and an F1 negative strain.

INTRODUCTION

Laboratories involved in the diagnosis of plague have frequently used immunofluorescence (IF) tests to detect *Yersinia pestis* organisms in animal or human specimens, or to confirm the identity of isolated bacteria. *Y. pestis* has a number of virulence determinants including the 'fraction 1' protein–lipid–polysaccharide complex that makes up the bacterial capsule (Bennett & Tornabene, 1974) and is specific for this species. Winter & Moody (1959; see also Moody & Winter, 1959) first reported the IF staining of *Y. pestis*, using rabbit antisera raised against killed whole cells of a virulent encapsulated strain. The specificity of this anti-*Y. pestis* reagent was verified with 100 strains of other bacterial species, including 19 strains of the closely related *Y. pseudotuberculosis*. By inoculating bacterial isolates into mice and subsequently staining mouse tissue impression smears with the fluorescent antibody these authors achieved diagnosis of plague 48–72 h earlier than possible by secondary isolation and agglutination tests.

Since then, IF tests based on polyclonal antibodies prepared against purified fraction 1 (F1) antigen (Baker *et al.* 1952) or more usually against live avirulent (but encapsulated) strains, especially *Y. pestis* A1122, have been used extensively in the investigation of wild rodent plague and domestic rat plague. Kartman (1960) was the first to report the use of IF for direct detection of plague bacteria in animal carcasses found in the wild, ie the bacteria were detected without intermediate isolation or animal inoculation procedures. Hudson *et al.* (1971) examined 354 fox squirrels found dead, and tested for plague bacteria by direct IF staining of smears from the carcass and also by mouse inoculation and subsequent culture for agglutination, phage and IF tests. Sixty-two carcasses were found positive by IF, but only 38 of these could be confirmed by inoculation of mice. Of a further 135 carcasses deemed too putrified for animal inoculation, 19 were positive by IF. Attempting to detect *Y. pestis* in tissues of rodents infected in the laboratory, Shepherd *et al.* (1986) achieved a higher proportion of positive results by IF than by enzyme-linked immunosorbent assay (ELISA) or immunodiffusion tests for F1, or by complement fixation tests. The Centers for Disease Control of the US Public Health Service routinely use an IF method based on polyclonal anti-*Y. pestis* A1122 as a presumptive test for plague in clinical specimens (Barnes & Poland, 1983).

A mouse monoclonal antibody against F1 that was produced in the laboratory of one of us (JEW) was used in the ELISA that is now employed routinely in South Africa to detect circulating F1 in human plague cases (Williams *et al.* 1984; Williams *et al.* 1986). The specificity of this monoclonal antibody for the *Y. pestis* F1 antigen makes it attractive compared to polyclonal reagents which in principle could include antibodies against antigens common to the *Yersinia* genus, eg some of the outer membrane proteins (Bolin, Portnoy & Wolf-Watz, 1985; Mazza, Karu & Kingsbury, 1985; Carniel, Mazigh & Mollaret, 1987). The work of Mazza, Karu & Kingsbury (1985) suggests that some outer membrane antigens that can be

extracted with SDS and detected by immunoblotting stimulate antibody production. It is possible that the single well-documented case of the staining of cells of a *Y. pseudotuberculosis* isolate with polyclonal antisera to F1 as well as antisera to A1122 organisms (Quan *et al.* 1965) did not involve F1 but some other cell surface antigen(s). *A priori*, IF tests utilizing monoclonal antibody to F1 are likely to be less prone to false positives than tests based on polyclonal reagents. Unfortunately, the possibility of false negatives remains because the classical dogma that the *Y. pestis* capsule and thus the F1 antigen are essential for virulence is not completely valid. Non-encapsulated (F1 negative) *Y. pestis* mutants can cause chronic, lethal infections in laboratory rats and mice (Williams & Cavanaugh, 1983, 1984), and an F1 negative strain of *Y. pestis* has been implicated in an acute fatal human infection (Winter, Cherry & Moody, 1960). Given that a test for F1 based on this monoclonal antibody would be at risk from false negatives if used as the sole criterion for detection of rodent plague or human plague, the certainty of diagnosis would be much improved if this test were used in tandem with a test able to detect non-encapsulated *Y. pestis*. Experience from the use of a pair of such tests would also help to improve understanding of the epidemiology of F1 negative plague infections.

In the present study we have started to address this requirement by developing IF tests of two designs, one utilizing an anti-F1 monoclonal and the other based on polyclonal antibodies raised against whole bacteria. The tests were evaluated for the detection of F1 positive and negative *Y. pestis* organisms in culture and in tissues of infected mice.

MATERIALS AND METHODS

Organisms

The *Y. pestis* strains 195/P, M23NP, A1122, CPS-1 and CPS-2a were from the collection of the US Army Walter Reed Army Institute of Research. The Soemedang and Tjiwidej strains were kindly donated by the David Bruce Laboratory, East Everleigh, Marlborough, Wilts, UK. Other *Y. pestis* strains used were from the collection at the former Microbiological Research Establishment (MRE), Porton Down, Salisbury, Wilts. The *Y. enterocolitica*, *Y. pseudotuberculosis* and *Brucella* strains were kindly donated by Dr M. J. Corbel, of the MAFF Central Veterinary Laboratory, Weybridge, Surrey, UK.

Yersiniae were grown by several methods: (1) blood agar method I. Bacteria were grown on blood agar (Oxoid CM55, pH 6·8) at 37 °C for 48 h, usually in Roux bottles. Cells were harvested in phosphate-buffered saline (PBS: 0·1 M-NaCl, 20 mM sodium phosphate buffer, pH 7·7), inactivated by adding 1% formaldehyde and incubating for 7 days at 37 °C, washed sixfold in PBS, and stored in PBS containing 0·1% formaldehyde. (2) blood agar method II. Bacteria were grown on trypticase soya/soybean casein digest agar containing 5% sheep blood, pH 7·4, in 9 cm Petri dishes, at 37 °C for 48 h. To harvest, each Petri dish was inverted over a cotton pad soaked with chloroform at room temperature (*c.* 25 °C), for 5 min. Bacteria were then resuspended from the agar surface in 5 ml of PBS, using an L-shaped glass rod. Suspensions were used directly for immunofluorescence tests, or were washed and/or treated with 1% formaldehyde, as described in the text. (3) brain heart infusion (BHI) agar method I. *Y. pestis* were grown on BHI agar, pH

7·4, in Petri dishes for 48 h, then inactivated with chloroform before being removed in PBS. (4) BHI agar method II. *Y. pestis* were grown on BHI for 48 h, removed in 0·1% formaldehyde/PBS, and incubated for 1 h before use. Chloroform was not used. (5) *Y. pestis* were incubated in BHI broth, pH 7·4, for 48 h, then inactivated by adding formaldehyde to 1% and incubating at room temperature for at least 1 h before use. Where stated, *Y. pestis* were grown at 25 °C rather than 37 °C; little, if any, F1 is produced at the lower temperature (Fox & Higuchi, 1958).

Yersiniae grown by blood agar method I were tested for sterility, then used on the open bench. Other preparations of yersiniae had all been treated with chloroform and/or formaldehyde, but they were handled in an open fronted containment hood until they had been dried on microscope slides and fixed in methanol for 5 min.

Bacillus anthracis Vollum spores were grown and inactivated with formaldehyde as previously described (Phillips & Martin, 1982*b*). Other bacterial species were grown overnight in nutrient broth in shake flasks at 37 °C, inactivated with formaldehyde overnight, washed, and stored in formaldehyde/PBS. Total counts of these bacteria and of yersiniae grown on blood agar I were determined by phase contrast microscopy.

Virulence determinants had been established previously in each culture collection essentially by the methods recommended by the WHO (Bahmanyar & Cavanaugh, 1976); thus, FI production was detected by precipitin formation from colonies in antiserum-agar plates or in Ouchterlony gel diffusion agar plates.

Antibodies

Rabbit antibodies to formaldehyde inactivated *Y. pestis* MP6, Tjiwidej, or Soemedang strains were prepared by hyperimmunization, then fractionated to IgG by Na_2SO_4 precipitation and DEAE chromatography, and conjugated with fluorescein as previously described (Phillips & Martin, 1982*a*, *b*). The fluorescein/protein (F/P) ratios of the three conjugates F-aMP6, F-aTjiw, and F-Soem were 13·2, 15·5, and 16·0 μg/mg. Rabbit IgG to *Y. pestis* A1122 strain (IgG-aA1122) was a commercial salt precipitated fraction (Lederle). This was further purified on DEAE Sephacel before being conjugated with fluorescein: the F/P ratio of the conjugate F-aA1122 was 13·5 μg/mg.

Mouse monoclonal anti-F1 was the IgA preparation 3G8 earlier described (Williams *et al.* 1984; Williams *et al*, 1986), either used as ascitic fluid or after further fractionation with $(NH_4)_2SO_4$. Because this monoclonal was prepared from a secretor cell line, the IgA was contaminated with IgG, even after fractionation. Fluorescein conjugate F-3G8 was prepared from the $(NH_4)_2SO_4$ fraction, and was absorbed with acetone dried guinea-pig liver powder and clarified by centrifugation and membrane filtration; the F/P ratio was 10 μg/mg.

The fluorescein conjugated goat anti-rabbit IgG (H + L chains) and goat anti-mouse IgG (H + L chains) reagents were prepared in the Department of Viral Pathogenesis and Immunology in the US Army Medical Research Institute for Infectious Diseases (USAMRIID). These are designated F-GaRabbit and F-GaMouse respectively. The fluorescein conjugated sheep anti-rabbit globulin (F-SaRabbit) was the preparation described previously (Phillips & Martin, 1982*a*, *b*).

Antibodies were stored in aliquots frozen or lyophilized; for use, they were diluted in one of the two types of bovine serum albumin buffer stated below, and held at 4 °C.

Immunofluorescence (IF) reaction

Working dilutions of bacteria were prepared daily by dilution to approximately 2×10^6 bacteria/ml, in 2 mM phosphate buffer pH 7·8 or deionized water. Five μl of this bacterial suspension were dried on 3 mm diameter wells of multispot glass microscope slides, then fixed in acetone or methanol. In direct assays performed in phase I of this study, each well was incubated with 5 μl of fluorescein conjugate diluted to 100 μg/ml in bovine serum albumin buffer containing Brij 35 detergent (BSABR: 1% bovine albumin (Sigma), 0·1% Brij 35 (BDH) in PBS), for 30 min at room temperature. Slides were then washed three times in 0·1% Brij/PBS, once in deionized water, and were dried. In phase II direct assays, rabbit fluorescein conjugates were used at the reduced concentration of 25 μg/ml, and Tween 20 detergent was substituted for Brij in the dilution and wash buffers. When the conjugated monoclonal antibody F-3G8 was used, the incubation period was extended to 45 min. In indirect assays, bacteria were incubated first with unlabelled anti-bacterial IgG, then washed, dried, and incubated with F-SaRabbit or, in phase II, with F-GaRabbit, for 30 min; slides were then washed and dried. In indirect assays with monoclonal anti-*Y. pestis*, bacteria were incubated with the unlabelled monoclonal for 45 min, then with F-GaMouse for 45 min. Slides were stored in the dark until required for microscopy, when they were mounted in glycerol mountant containing bleach retardant (Citifluor: the City University, London). Fluorescence intensity of bacteria was scored subjectively on a scale from 0 (autofluorescence) to 7.

Blocking of antisera with fraction 1 antigen

Antibody conjugates were incubated with F1 antigen at a tenth of the antibody concentration, in the presence of 1% BSA, for 2 h at room temperature. This mixture was filtered through a 0·22 μm membrane filter (Millipore), then diluted for use in IF tests. To prevent unacceptable background staining when blocked conjugate preparations were used to stain yersiniae, bacteria dried on multispot slides were incubated with BSABR for 30 min, then washed with Brij/PBS and water, and air dried, prior to reaction with blocked antisera. Blocking with F1 prevented the staining of F1+ strains of *Y. pestis* by polyclonal conjugates used at a concentration of 100 μg/ml. That this effect was specific was indicated by the lack of interference with the staining of brucella bacteria incubated with anti-brucella conjugates in the presence of F1-blocked anti-*Y. pestis* conjugate.

Purification of fraction 1 antigen

A 100 μl sample of F1 antigen, prepared by the method of Baker *et al.* (1952), at a concentration of 2 mg/ml in 10 mM ammonium acetate buffer, was injected onto an TSK-G4000SW HPLC column, 600 × 7·5 mm (LKB Ltd). Protein concentration in the eluate was followed at 280 nm, and eluate fractions were lyophilized.

Infection of laboratory mice

Four-month-old Balb/c mice were inoculated subcutaneously with 0·2 ml of BHI broth containing approximately 5×10^4 *Y. pestis* 195/P, CPS-2a, or EV76 that had been grown at 25 °C for 18 h. *Post mortem*, spleen and blood smears were made on multispot microscope slides, allowed to dry, and fixed in methanol for 5 min. Spleen impressions and blood samples were also streaked on blood agar to confirm the presence of *Y. pestis* by isolation of bacteria and testing with specific bacteriophage.

ELISA for fraction 1 antigen

Fraction 1 concentrations in formaldehyde treated *Y. pestis* suspensions (approximately 10^{10} organisms/ml) were measured essentially by the ELISA method described by Williams *et al.* (1986). The ELISA could detect F1 down to concentrations of 6 ng/ml, as shown by concurrent tests of a standard F1 antigen solution.

RESULTS

Phase I: *Choice of polyclonal antisera — specificity for* Y. pestis

Fluorescein conjugated IgG fractions of hyperimmune antisera against *Y. pestis* strains were evaluated in direct IF assays of *Y. pestis* and *Y. enterocolitica* organisms grown on blood agar I and stored in 0·1% formaldehyde/PBS for months and in some cases years. As may be seen in Table 1, each of the four conjugates tested reacted to a broadly similar degree with F1+ strains and F1− strains, except that two conjugates reacted less well with the F1− strain M16. In view of the advantage possessed by avirulent *Y. pestis* strains in obtaining high titre antibodies by hyperimmunization, F-aA1122 and F-aTjiw were chosen for further study in preference to conjugates derived by immunization with the fully virulent MP6 strain. However, attempts were made to prepare from the F-aMP6 conjugate an IF reagent able to react specifically with F1−, VW+ strains as represented by MRE 2492. Progressive absorption with any of the F1+, VW− strains Soemedang, Tjiwidej, MRE 1197 or MRE 2518 resulted in loss of activity towards MRE 2492 earlier than the activity towards F1+ strains.

The cross-reaction of F-aTjiw with 12-15% of the bacteria in preparations of the *Y. enterocolitica* serovar O 8 strain Carter WA was investigated by selective absorption of conjugates with F1− *Y. pestis* strains. The number of *Y. enterocolitica* organisms immobilized on replicate microscope wells was determined by staining with anti-*Y. enterocolitica* conjugate. Absorption with *Y. pestis* MRE 2492 reduced the proportion of *Y. enterocolitica* stained from 13% to 3%, and the brightness of the stained cells was reduced from 2 to 1 unit. The degree of staining of *Y. pestis* Soemedang preparations (F1+ organisms) was not reduced by this absorption. Blocking of the conjugate by prior incubation with F1 antigen reduced the proportion of bacteria stained to 1%, again with scores of 1 unit. However, when the F1 preparation was fractionated by HPLC, the early column fractions believed to contain the F1 did not inhibit the staining of *Y. enterocolitica*, but the later fractions caused partial inhibition. This suggests that other *Y. pestis* antigens contaminating the original F1 preparation and not

Table 1. *Immunofluorescence reaction of* Y. pestis *and* Y. enterocolitica *with polyclonal anti-*Y. pestis *conjugates*

Test organism	F1 phenotype	IF score F-aMP6	F-aA1122	F-aTjiw	F-aSoem
Y. pestis					
M39	+	4	6	4	2
139L	+	3	4	2	1
A29	+	3	4	3	2
MP6	+	4	6	3	2
2518	+	6	6	5	3
Soemedang	+	3	4	2	2
Tjiwidej	+	3	5	3	2
Bryans	−	4	6	3	1
M16	−	1	2	2	1
M18	−	2	4	2	1
MRE 1330	−	3	4	2	2
TRU	−	2	4	2	1
Y. entero-colitica					
	Serovar				
Carter WA, 2705	O 8	NT	1*	2*	NT
296/68	O 9	NT	0	0	NT
IPP† 864	O 3	NT	0	0	NT
IPP 383	O 9	NT	0	0	NT
CVL† 3045	O 7	NT	0	0	NT
CVL 3051	O 7	NT	0	0	NT
CVL 3059	O 6,30	NT	0	0	NT
CVL 3066	O 2	NT	0	0	NT
CVL M8542	O 3	NT	0	0	NT

Bacteria were grown by blood agar method I.
* About 12% of bacteria were stained; the remainder were scored as 0.
† IPP: Institut Pasteur de Paris. CVL: Central Veterinary Laboratory, Weybridge.
NT, not tested.

F1 itself had caused the inhibition, and the presence of F1 antigen on the *Y. enterocolitica* cells is not necessarily implied. Consistent with this, preliminary indirect assays using the ascites monoclonal anti-F1 reagent gave no staining of this *Y. enterocolitica* strain, though the F1+ *Y. pestis* strains cited in Table 1 all stained with the monoclonal.

The F-aA1122 conjugate reacted to a similar extent with this *Y. enterocolitica*, as did the IgG preparations of anti-A1122 and anti-Tjiw when used in indirect IF assays. This indicates that the cross-reaction was not an artefact caused by damage to the antibody during conjugation.

Selective absorption with F1− *Y. pestis* strains was used in an attempt to make a polyclonal reagent specific for F1+ strains of plague. Absorption of F-aTjiw conjugate with *Y. pestis* MRE 1330 reduced the fluorescence intensity of F1+ strains by about 1 unit, but reduced the scores for F1− *Y. pestis* strains virtually to autofluorescence levels. However, such absorption did not affect the staining of the minor population in *Y. enterocolitica* serovar O 8 preparations. In view of the persistent cross reactions with this *Y. enterocolitica* serovar, the anti-F1 monoclonal was considered a better choice for investigation as an IF reagent for F1 positivity in bacteria.

Table 2. *Immunofluorescence reaction of* Y. pestis *with polyclonal and monoclonal antibodies in direct and indirect assays*

Y. pestis strain	F1 phenotype	IF score* F-aA1122	F-aTjiw	IgG-aA1122 + F-GaRabbit	F-3G8	IgA-3G8 + F-GaMouse
A1122	+	5-7	4 (6) B	4, 5B	2, 3 (4, 5)	2–4 (5, 6)
MRE 1197	+	5, 6 (7)	3, (4, 5) B	4, 5B	1–3 (4, 5)	2–4, 6
MRE 2518	+	4–6	3–5	3, 4 (5)	3 (4)	3, 4 (5, 6)
Soemedang	+	5, 6 (7)	2, 3B	3–5	1, 2 (3, 4)	4, 5B
Tjiwidej	+	(5) 6, 7	3–6B	5, 6	2, 3 (4, 5)	3, 5, 6B
MRE 2482	−	4, 5	2, 3B	3, 4	0	0
MRE 2492	−	4	2B	3	0	0
MRE 1330	−	5, 6	3B	4, 5	0	0
M23 NP	−	5 (6)	0	1–5	0	0

Bacteria were grown by blood agar method II, without treatment with formaldehyde.

* Multiple IF scores, e.g. 3-5, indicate approximately equal numbers of bacteria of each brightness value within this range, except that scores in parentheses indicate that less than 10% of cells stained to this degree. The designation B denotes substantial staining of the background.

Indirect IF assays based on anti-A1122 IgG and anti-Tjiw IgG, and direct assays based on F-aTjiw, were screened against formalin inactivated preparations of the following bacterial strains, and no cross reactions were found: *Brucella abortus* 544 (A+M−), *Br. melitensis* 16M (A−M+), *Br. suis* 40 (A+M+), *Br. ovis* 63/290 (A−M−), *Br. neotomae* (A+M−); *Francisella tularensis* LVS, FAM, SCHU4, HN63, TN53, MRE 614, MRE 617, MRE 618, 308/67; *Pseudomonas pseudomallei*; *Salmonella morehead*, *S. sobrenga*, *S. typhimurium*; *Bacillus anthracis* Vollum spores; *Serratia marcescens* UK8; *Escherichia coli* MRE 162 (O 8·K 9), MRE 160; *Shigella shiga*; *Proteus vulgaris*.

Phase II: *Staining of F1+ and F1−* Y. pestis *with polyclonal and monoclonal reagents*

In phase II, USAMRIID goat anti-rabbit IgG conjugate was used instead of the F-SaRabbit reagent used above and in earlier studies (Phillips & Martin, 1982*b*) because it did not suffer from minor direct reactions with the surface of formalized F1+ *Y. pestis* strains in control assays. Results of direct and indirect IF assays based on polyclonal and monoclonal anti-*Y. pestis* reagents, for bacteria grown on blood agar II may be seen in Table 2. Even though the reaction conditions had been optimized in indirect polyclonal assays to provide maximal staining of test bacteria whilst minimizing the staining in control assays lacking the first antibody, the staining intensity achieved in indirect assays was always less than in the analogous direct assays. There was more background staining in indirect assays, leading to a reduction in contrast in visualizing stained bacteria. In the monoclonal IF assays, the indirect format gave IF scores about 1 unit above direct assay scores, but again the increased staining of background, presumably

including cell-free capsular material, reduced the contrast for bacteria themselves. In the two types of direct polyclonal assay, the F-aTjiw reagent gave lower IF scores than F-aA1122, with considerable background staining. Increasing the concentration of F-aTjiw did not increase the bacterial staining intensity. Both direct polyclonal reagents stained F1+ and F1− cells, though IF scores for the latter were generally lower when F-aTjiw was used. Preparations of the Soemedang strain consistently gave IF scores in direct assays with the monoclonal antibody that were lower than for other F1+ strains. The striking difference between the polyclonal and monoclonal results was the greater heterogeneity of staining with monoclonal reagents, these giving a skew distribution of fluorescence with a preponderance of poorly stained cells.

Washing these bacterial preparations threefold in PBS did not affect the IF result; in particular, washing did not reduce the proportion of bacteria stained well by F-3G8. The effect of formalization was also investigated. Immediately after harvesting the bacteria, an aliquot of each cell suspension was treated with 1% formaldehyde; scores in IF tests performed the next day or 18 days later were no less, and in some cases marginally higher, then scores for untreated specimens. F1+ strains grown in BHI broth and inactivated with formaldehyde gave IF scores about 1 unit lower than for the blood agar II growths. When *Y. pestis* were grown by BHI agar method I a very high level of background staining occurred in the IF tests, preventing accurate assessment of the brightness of the bacteria. That this background staining was the result of reaction of conjugate with cell-free capsule was suggested by the absence of background when the same strains were grown on BHI agar at 25 °C, because FI antigen would not be produced at the lower temperature. The F1+ bacteria grown at 25 °C stained well with F-aA1122, but were not stained at all by F-3G8. Four F1+ strains that were grown by BHI agar method II, i.e. washed off in 1% formaldehyde/PBS without prior chloroform treatment, suffered much less from background staining in IF tests.

The performance of the F-aA1122 reagent with bacteria grown at 37 °C and 25 °C was also investigated for the F1− strains CPS-1 and CPS-2a earlier reported by one of us to cause chronic lethal infection in laboratory rodents (Williams & Cavanaugh, 1983, 1984). As shown in Table 3, for cells grown on blood agar II at 25 °C the IF score was actually higher than at 37 °C. As expected, none of these F1− cultures stained with the F-3G8 monoclonal. A microphotograph from a polyclonal IF assay of CPS-2a cells grown on blood agar II is shown in Figure 1a. Cells of the fully virulent *Y. pestis* strain 195/P reacted with F-aA1122 when grown at either temperature, and with F-3G8 when grown at 37 °C.

Measurement of fraction 1 antigen in bacterial suspensions

ELISA titres of F1 antigen varied by no more than a factor of 4 for the F1+ *Y. pestis* strains grown by any one method at 37 °C, but F1 titres for 48 h cultures grown on agar (blood agar method II or BHI agar method I) were markedly greater than for BHI broth preparations, in the range 64000–256000 ng/ml compared to 250–1000 ng/ml. F1+ strains grown at 25 °C gave F1 titres at the assay sensitivity limit of 6 ng/ml, and given this limit it is concluded that discrimination between F1+ and F1− genotypes by ELISA of 37 °C cultures

Table 3. *Effect of growth temperature on reaction of F1+ and F1−* Y. pestis *with polyclonal and monoclonal anti-*Y. pestis *conjugates*

Y. pestis strain	F1 phenotype	Growth temp.°C	IF score* F-aA1122	F-3G8
CPS-1	−	37	4, 5	0
	−	25	6	0
CPS-2a	−	37	4, 5	0
	−	25	5, 6	0
195/P	+	37	(3) 4–6	(2) 3, 4
	−	25	5, 6	0

Bacteria were grown by blood agar method II, and treated with formaldehyde.

* Multiple IF scores, e.g. 3–5, indicate approximately equal numbers of bacteria of each brightness value within this range, except that scores in parentheses indicate that less than 10% of cells stained to this degree.

Table 4. *Identification of F1+ and F1−* Y. pestis *in mouse spleen and blood smears by reaction with polyclonal and monoclonal anti-*Y. pestis *conjugates*

Infecting *Y. pestis* strain	FI phenotype	Specimen	IF score* F-aA1122 >1000 orgs	F-aA1122 <10 orgs	F-3G8 >1000 orgs	F-3G8 <10 orgs
195/P	+	Spleen	5	–	3, 4	–
		Blood	5	–	2, 3	–
EV 76	+	Spleen	5	–	3, (4)	–
		Blood	–	6	–	4
CPS-2a	−	Spleen	3	–	–	0
		Blood	–	4	No orgs seen	

* Multiple IF scores, e.g. 3, 4, indicate approximately equal numbers of bacteria of each brightness value within this range, except that scores in parentheses indicate that less than 10% of cells stained to this degree. The numbers of organisms stated are the fluorescent bacteria counted on the entire 3 mm diameter multispot slide well.

should be attempted after growth on agar rather than in broth. The present F1− strains tested after growth on blood agar at 37 °C gave F1 titres of $\leqslant 6$ ng/ml, except for strain CPS-1 which gave a titre of 100 ng/ml.

Identification of Y. pestis *organisms in mouse spleen and blood*

Having determined the specificity of the F-aA1122 and F-3G8 conjugates for F1+ and F1− *Y. pestis* organisms cultured *in vitro*, the potential of these reagents for diagnosis of plague infections was investigated using laboratory mice. In preliminary experiments involving F-aA1122 at the concentration found satisfactory for screening isolates of *Y. pestis*, i.e. 25 μg/ml, it appeared that the large amount of cell-free *Y. pestis* antigenic material evident in the host tissues, including capsule (Walker, 1962), had reduced the free concentration of specific antibody below the level needed for adequate staining of bacteria. This effect was overcome by doubling the concentration of conjugate.

Mice were infected with the fully virulent *Y. pestis* strain 195/P, with the F1+ vaccine strain EV76, or with the F1− strain CPS-2a; all animals died within 7

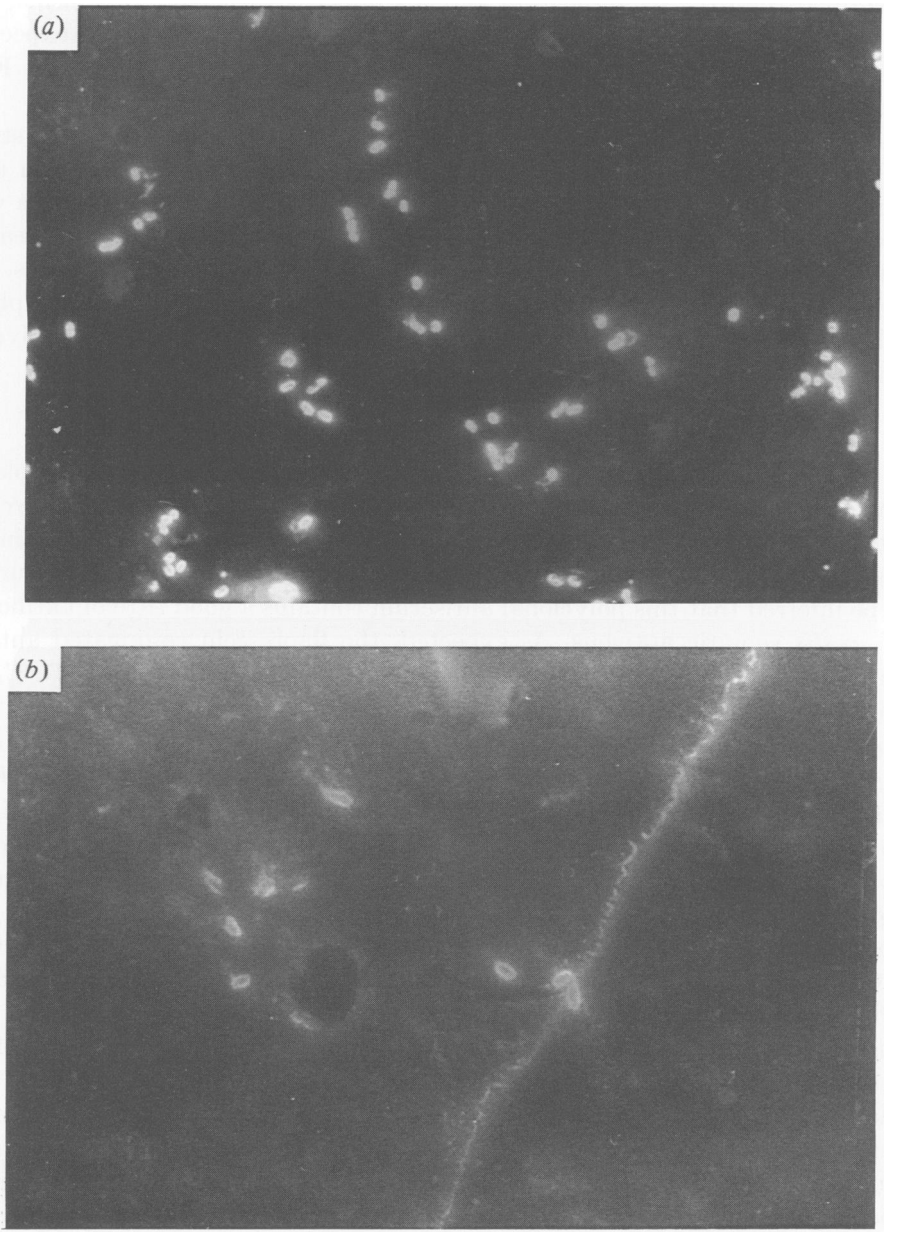

Fig. 1. Microphotographs of *Y. pestis* organisms stained with polyclonal and monoclonal conjugates. (*a*) CPS-2a grown by blood agar method II and treated with formaldehyde, then stained with F-aA1122. Bacteria were scored as 5 units. (*b*) 195/P in mouse spleen smears stained with F-3G8. Bacteria were scored as 3 and 4 units.

days. The IF scores when smears of spleen or blood were tested with F-aA1122 or F-3G8 are shown in Table 4. After lethal infection with any of these three plague strains large numbers of bacteria could be visualized in spleen smears, several thousand bacteria on each 3 mm diameter microscope slide well (see Fig. 1*b*). Bacteria in the spleen of animals infected with the F1− strain CPS-2a were less

well stained than the bacteria from animals infected with F1+ organisms, and were also less bright than CPS-2a cells grown *in vitro* (see Table 3). As expected, the monoclonal F-3G8 only detected bacteria in spleen after infection with F1+ strains.

The IF tests indicated a massive bacteraemia in animals infected with strain 195/P, but very few stained bacteria were observed in blood smears from mice inoculated with EV 76. Animals infected with CPS-2a also seemed to have a very limited bacteraemia, and only F1− bacteria were observed. Bacteria were readily isolated from all these blood and spleen samples by culture on blood plates, and the identity of cultures as *Y. pestis* was verified in plaque tests with specific phage on BHI agar. Immunofluorescence and culture tests for *Y. pestis* were negative for normal mice.

DISCUSSION

It is clear that hyperimmunization with live bacteria of the avirulent plague strain A1122 can be used to produce direct IF reagents which perform well in identifying F1+ and F1− plague strains grown *in vitro* at 25 or 37 °C. Since a capsule is not produced (or at least not assembled) at the lower temperature, it may be inferred that this polyclonal antiserum contains a good titre of antibodies to *Y. pestis* somatic antigens. As expected, the fluorescein conjugated anti-F1 monoclonal, F-3G8, reacted with F1+ strains when these were grown at 37 °C, but did not stain bacteria grown at 25 °C. The monoclonal also did not cross react with *Y. enterocolitica* biovar 2, serovar O 8, known to carry the *Yersinia* V and W antigens. The cross reaction of the polyclonal F-aA1122 with this *Y. enterocolitica* strain is not surprising in view of the known commonality of structural antigens among the yersiniae. Our inability to prepare a polyclonal reagent specific for *Y. pestis* by selective absorption further points to the advantage of having a monoclonal antibody specific for fraction 1 antigen.

Conditions were readily found for culturing suspected plague isolates and harvesting the bacterial colonies for IF tests so as to give sufficient F1 production for bacterial detection by F-3G8 but not so much cell-free F1 material that background staining obscures the stained bacteria. The tentative conclusion from varying the harvesting procedure for bacteria grown on BHI agar is that formaldehyde treatment can reduce the staining of cell-free capsular material on the microscope slide; we have no evidence how formalization of proteins might interfere with the chain of events leading to high background staining. The results for bacteria grown on blood agar indicate that formalization also tends to increase effective binding of polyclonal and monoclonal antibody per bacterium. Excessive staining of background is likely to have two deleterious effects on IF assays. The first is the reduction of the contrast of stained bacteria purely as an optical effect. Secondly, reaction of antibody with cell-free fraction 1 antigen and other background material may reduce the concentration of specific antibodies below the level needed for maximum staining of bacteria; we have recently found that the brightness of *Bacillus anthracis* spores and *Escherichia coli* in IF tests using polyclonal reagents was readily reduced by increasing the bacterial concentration relative to the conjugate concentration (Phillips, Martin & Capey, 1987). Absorption of polyclonal antisera with fraction 1 antigen and rodent tissue

extracts should be considered in order to improve further the specific staining of plague somatic antigens and thus enhance the staining contrast in spleen smears. If care is taken to optimize staining for bacterial somatic antigens such polyclonal conjugates may have potential for staining plague organisms in the midgut of infected fleas living in the temperature range 20–25 °C, in spite of the lack of success achieved by Hudson, Kartman & Prince (1966) using antisera to A1122.

Our experience of Indian ink staining of *Y. pestis* has been that the apparent cell 'envelope' delineated by the ink is several times larger than the somatic bacterium when *Y. pestis* are grown on blood agar or chocolate agar, but tends to be little larger than the cell after growth on BHI agar. Preliminary studies with the Soemedang and Tjiwidej strains indicated that this variation in the size of the Indian ink-exclusion envelope did not affect the degree of staining with either polyclonal or monoclonal reagents. We infer that the anti-F1 monoclonal has reacted only with F1 sites on the underside of the capsule, close to the cell surface; the outer boundary of the capsule does not appear to have been stained.

The marked heterogeneity of staining of individual F1+ *Y. pestis* organisms with the monoclonal antibody may be due to phenotypic differences in the amount of capsule produced or in the fragility of the capsule during cell harvesting, eg. for cells in different growth stages, or may be the result of differences in the environment at the cell-capsule boundary. The fact that heterogeneity of staining did not occur to the same extent when *Y. pestis* were stained with polyclonal reagents suggests that variation in the permeability of the capsule to antibody is not an important factor. The hypothesis that all bacteria in these populations have F1+ genotypes could be tested by the use of a DNA probe. Flow immunofluorescence and cell sorting would be ideal for investigating the phenotypic and genotypic basis of the production of the F1 antigen (Phillips & Martin, 1985; Phillips, Martin & Capey, 1987).

The ELISA measuring fraction 1 antigen in solution has a high sensitivity that should allow investigation of low levels of F1 production undetected by classical precipitin tests, and thus the basis of the 100 ng/ml of F1 recorded in assays of CPS-1 cells grown on agar could be further examined. Strain CPS-1 is a heterogeneous *Y. pestis* population from a rat's bubo, in which some organisms may produce small amounts of F1 antigen (J. E. W., unpublished observations). In contrast, the other F1 negative strains used in this study are clones isolated by picking single colonies from agar media.

Even when the fluorescence intensity of stained *Y. pestis* was greater in indirect assays than in direct ones, increased background staining prevented overall improvement in the bacterial contrast. We earlier found little amplification of staining intensity in indirect assays of bacillus spores; lack of amplification may be a reflection of steric constraints on the packing of indirect antibody molecules as a result of high antigen density (Phillips & Martin, 1982*b*). When the classical hypothesis that transition to the indirect format should be accompanied by major amplification of the assay signal (Nairn, 1976) is not realised, direct antibody methods for antigen detection are to be preferred because as well as being quicker to perform there is no inherent risk of the anti-species second antibody reacting directly with the bacterial surface. The advantage of using conjugated monoclonal antibodies to study plague in rodents is that this avoids the specificity

complications arising from the use of indirect anti-mouse reagents to detect mouse monoclonal reagents in rodent tissues. Direct tests based on the polyclonal and monoclonal antibodies described in this report should be equally applicable to epidemiological studies of plague in rodents or humans.

When using the direct polyclonal and monoclonal reagents in tandem we found it convenient to assess first the fluorescence result with F-aA1122; the bacteria were for the most part brighter than when stained with the F-3G8 monoclonal, and thus easier to find. From the numbers of positive bacteria found in the polyclonal test, the question of their F1 phenotype could easily be addressed when the replicate sample tested with F-3G8 was examined. Thus, in a diagnostic context where many specimens require examination, screening with a polyclonal conjugate and following up with IF tests of the reactors using the anti-F1 monoclonal conjugate would be an efficient means of obtaining firm diagnoses by detecting *Y. pestis* in rodent carcasses or human clinical materials (bubo aspirates, blood, sputum, autopsy specimens). While the monoclonal is clearly the conjugate of choice for specificity, there remains the potential for missing F1− *Y. pestis* organisms. Thus, IF tests with both monoclonal and polyclonal conjugate should be performed if possible. Although few natural infections with F1− plague bacilli are documented, additional examination of the bacteria isolated is indicated when they react with polyclonal conjugate but not with the monoclonal, especially when clinical signs suggest plague. Given the availability of bleach retardants (Phillips, 1984) which allow multiple examinations of microscope slides and also facilitate colour photography of fluorescent bacteria, even laboratory staff inexperienced with immunofluorescent techniques should find it easy to achieve a high diagnostic success rate using these IF reagents.

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