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Serodiagnosis of human plague by an anti-F1 capsular antigen specific IgG/IgM ELISA and immunoblot

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SUMMARY

Plague is a re-emerging disease endemic in at least 24 countries. Non-endemic countries should be able to confirm plague to prevent outbreaks due to imported cases. We established a combination of a IgG/IgM screening ELISA and a confirmation immunoblot employing F1 capsular antigen (CA) for the serodiagnosis of plague in countries where yersiniosis is present. The ELISA and the immunoblot assay showed a specificity of 96·1% and 100% among sera from healthy German blood donors. This group had a seroprevalence of 39% of anti-yersinia outer protein (YOP) antibodies obviously caused by previous *Y. enterocolitica* infection. The ELISA detected anti-F1 CA antibodies in 22 and the immunoblot in 20 out of 26 sera of plague vaccinees. Five control sera from bacteriologically confirmed plague cases from Madagascar reacted positively. It can be concluded that anti-YOP antibodies do not affect assays based on purified F1 CA.

INTRODUCTION

Plague is an acute, life-threatening bacterial infection caused by *Yersinia pestis*. Once plague is clinically suspected, a laboratory confirmation should be obtained as quickly as possible to ensure proper treatment and prevention of secondary cases. Bacterial isolation is still considered as the 'gold standard', although recovery of *Y. pestis* is often impossible due to inadequate sampling or prior treatment of patients with antibiotics. In these cases, serological testing might be the only way to retrospectively confirm the diagnosis of plague [1].

Purified F1 capsular antigen (CA) plays a major role in all serological tests used so far [2–7]. The passive haemagglutination assay (PHA) is the most widely employed method to detect F1 CA specific antibodies as it is recommended by the World Health Organization [2]. Although easy to perform, there are no standards for the protocol or antigen preparation. Therefore other techniques, mostly enzyme-linked immunosorbent assays (ELISA) have been introduced. Compared to the PHA these tests give more reliable results. ELISAs were also used to evaluate vaccine efficacy and experimental plague in laboratory animals [5, 8–11]. However, these ELISAs were evaluated in populations with a high prevalence for plague and in a geographical area where infections with the closely related species *Y. pseudotuberculosis* and *Y. enterocolitica* do not occur [3, 9, 10, 12–14]. In mainland Europe, yersiniosis caused by *Y. entero-*

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colitica is a common infectious disease. It is sometimes followed by severe post-infectious sequelae such as erythema nodosum or reactive arthritis. Immunoblot techniques (IB) based on the use of yersinia outer proteins (YOP) have been introduced to study and diagnose yersiniosis [15]. Seroprevalence up to 40% of healthy Europeans have been reported by several investigators [16–19].

The aim of this study was to evaluate a F1 CA ELISA and F1 CA immunoblot for the diagnosis of plague in a non-endemic area.

MATERIALS AND METHODS

F1 capsular antigen and whole cell antigen

Purified F1 CA from strain *Y. pestis* EV 76 was kindly provided by the Russian State Research Centre of Applied Microbiology, Obolensk, Russia.

Test sera

For evaluation of the F1 CA ELISA and the F1 CA immunoblot, different groups of test sera were used. 103 sera from healthy German blood donors with no history of *Y. pestis* infection or plague vaccination were obtained from the Central Institute of the German Armed Forces, Koblenz, Germany in 1998. Twenty-six sera from laboratory personnel vaccinated against plague with inactivated Plague Vaccine USP (Greer Laboratories, Lenoir, NC, USA) or Plague Vaccine (CSL Limited, Parkville, Australia) were collected from 1996–1998. All vaccinees received the number of doses recommended by the producers. The Cutter vaccine is normally given as 1 ml injection at time 0, followed by a 0·2 ml booster at 1–3 months and again at 6 months. Six monthly boosters are recommended for at risk personnel. The CSL vaccine is given as 0·5 ml doses at time 0 and 1–4 weeks, followed by 6 monthly boosters. Furthermore, five convalescent sera from *Y. pestis* culture-proven plague cases (male, aged from 9–55 years) were investigated. The sera were collected during the Mahajanga, Madagascar plague outbreak from August to September 1998. All patients were treated just after the clinical diagnosis of plague (bubonic plague with cervical, inguinal or axillary buboes) and all patients recovered subsequently. Three sera from serologically confirmed yersiniosis patients (positive in the complement fixation test and high IgG and IgA ELISA antibody titers against YOPs) were supplied by Mikrogen, Munich, Germany. Sera were stored at −20 or −80 °C till use. All specimens were tested simultaneously by ELISA and immunoblot.

ELISA

For the IgG/IgM F1 CA ELISA, 96 well microtitre plates (PolySorp, Nunc, Germany) were coated with purified F1 CA in PBS pH 7.2 at 4 °C overnight (0·5 μg/ml, 100 μl per well). Plates were washed four times using PBS/Tween 20 0·1% and then incubated with blocking buffer (1% (w/v) bovine serum albumin (BSA) in Tris-HCl, 50 mmol/l; NaCl, 150 mmol/l; (all Sigma, Germany); pH 7·5) at 37 °C for 1 h. After washing, serum samples were diluted 1/500 in PBS, transferred to the plate (100 μl/well) and incubated at 37 °C for 1 h. Plates were washed again and incubated with a mixture of rabbit anti-human IgG and anti-human IgM horseradish peroxidase conjugate (HRP) (Dako, Germany) at a final dilution of 1/4000 for an additional hour (37 °C). After four washing steps, tetramethylbenzidine/peroxide (TMB, Seramun, Germany) was added as substrate and incubated for 15 min at room temperature. The reaction was stopped by adding 25 μl H_2SO_4 (2·5 mol/l) and results were determined by measuring absorbance at 450 nm with an automated microplate reader. In each experiment a substrate control (blank), a negative control and two positive controls (high and low absorbance) were run in parallel with the test samples; all tests were performed in duplicate. Samples giving an absorbance above 0·350 were considered as positive (cut-off). The cut-off was calculated as the mean OD plus 3 S.D. of the correctly identified samples of the healthy blood donor group.

Immunoblotting

Immunoblotting of F1 CA was performed according to following protocol. 100 μl F1 CA in distilled water (i.e. 10 μg pure F1 CA) were mixed with 2 × Tris-glycine sample buffer (Novex, Frankfurt, Germany) and 10 μl β-mercaptoethanol, incubated for 10 min in a boiling water bath and then loaded onto a Tris-glycine 2D preparative 4–20% precast PAGE gel. The XCell II Mini-Cell and the XCell Blot Module with buffers supplied by Novex was used for electrophoresis and blotting. SeaBlue weight markers were used to indicate molecular weight. After electrophoresis and transfer to a nitrocellulose membrane (Novex), the membrane was incubated in 10% skimmed milk in TBS at 4 °C overnight. The membrane was dried and cut into strips 5 mm wide.

Table 1. *Reactivity of sera tested in F1 CA ELISA and F1 CA Immunoblot*

	F1 CA ELISA		F1 CA Immunoblot	
Group	Positive	Negative	Positive	Negative
Healthy blood donor	4	99	–	103
Vaccinee	22	4	20	6
Yersiniosis patient	–	3	–	3
Plague patient	5	–	5	–

Controls consisted of rabbit anti-*Y. pestis* hyperimmune serum (Biotrend, Köln, Germany) 1/10000 diluted in PBS (pH 7·4) and goat anti-rabbit Igs HRP conjugate (Dako). Human sera were investigated using a mixture of rabbit anti-human IgG/IgM HRP conjugates (Dako). Conjugates were diluted 1/500 in washing buffer. The strips were incubated in 2·0 ml washing buffer (Mikrogen, Munich, Germany) containing 20 μl sample serum. After incubation for 1 h at 37 °C, strips were washed twice in washing buffer for 10 min each. The antibody conjugate reaction was detected with DAB and H_2O_2 according to the recommendations of the supplier (Mikrogen). Substrate reaction was stopped after 5 min and strips were washed in distilled water. Strips were dried and read after 2 h. A brownish or purple precipitate visible by eye corresponding to the signal of the positive control strip was reported as positive (+). Weaker but still visible reactions were termed +/−, stronger reactions ++ or +++.

The Immunoblot *Yersinia* IgG/Immunoblot IgM-IgA was used according to the manufacturer's (Mikrogen) recommendations. The IB detects antibodies directed against YOPs from *Y. enterocolitica* and *Y. pseudotuberculosis* although the assay is based on YOPs from *Y. enterocolitica*.

RESULTS

IgG/IgM F1 capsular antigen ELISA

All five positive control sera from culture-proven plague cases gave a positive signal with absorbance (A_{450}) ranging from 0·360 to 0·794 (Table 1). Employing a cut-off level of 0·350, the F1 CA ELISA identified 99 from 103 samples of the healthy blood donor group (no history of plague, no vaccination) as negative giving a specificity of 96·1% (Table 1). None of the sera of yersiniosis patients ($n = 3$) were reported as positive. When sera from 26 persons vaccinated against plague were tested, in 22 samples antibodies reacted with the F1 CA, representing 84·6% of all vaccinees included (Table 1). The mean absorbance in the vaccinees group was higher than in the plague patients group (range 0·407–2·507; data not shown).

F1 capsular antigen immunoblot

The F1 CA IB identified all five sera from the convalescent plague patients as positive. No serum sample of a healthy blood donor ($n = 103$) or of a yersiniosis patient ($n = 3$) produced a signal on the F1 CA IB strip. Only 20 out of 26 serum samples from vaccinees contained antibodies reacting with the F1 CA on the strip (Tables 1).

Immunoblot *Yersinia* IgG/Immunoblot IgM-IgA

A significant number of healthy blood donor sera contained anti-YOP IgG ($n = 36$), IgA ($n = 3$) and IgM ($n = 4$) antibodies. One serum contained anti-YOP antibodies of all three Ig subtypes. Two sera from the healthy blood donor group and two sera from the vaccinee group showing an extinction value over the cut-off of the F1 CA ELISA but no signal on F1 CA IB strips were diagnosed as negative (Table 2). There was also no visible band when sera from plague patients ($n = 5$) were tested (Table 1). However, the three sera of yersiniosis patients contained antibodies to at least seven YOPs and were consequently diagnosed as positive (Table 2).

DISCUSSION

There is no commercially available test for the diagnosis of plague that is standardized or internationally approved. This lack is a serious problem for the health authorities of countries in non-endemic areas who have to ensure that imported cases of this dangerous and highly communicable disease are speedily identified and quarantined. In this study we evaluated the combination of a F1 CA IgG/IgM

Table 2. *Anomalous reactions in the F1 CA ELISA are not caused by the presence of anti-yersinia outer protein antibodies*

Group	F1 CA ELISA* (OD)	Y.e.IB	F1 CA IB
HBD 008	0·410	+	−
HBD 018	0·537	−	−
HBD 050	0·620	+ + +	−
HBD 080	1·545	−	−
Vaccinee 18	0·926	−	−
Vaccinee 22	1·820	−	−
Yersiniosis patient 1	0·100	+	−
Yersiniosis patient 2	0·152	+ + +	−
Yersiniosis patient 3	0·170	+ + +	−

* The OD cut-off value was calculated to be 0·35. HBD, healthy blood donor; CA, capsular antigen; Y.e.IB, Immunoblot Yersinia IgG/Immunoblot IgM-IgA. (−) negative; (+) positive.

screening ELISA and a F1 CA immunoblot for the serodiagnosis of plague in a non-endemic country but where yersiniosis is endemic.

ELISAs employing F1 CA have already been used in the diagnosis of plague in endemic countries [2–4, 6, 7, 12, 13]. We investigated samples from a group of healthy blood donors in a non-endemic area (Germany) with no history of plague or vaccination. Four sera were diagnosed as positive (Table 2). The sera may contain anti-YOP antibodies arising from a previous *Y. enterocolitica* or *Y. pseudotuberculosis* infection and reacting with YOP antigens co-precipitated with the F1 CA preparation although this is not very likely as *Y. pestis* cells used for F1 CA production were not cultivated under Ca^{2+} deficient conditions. However, no additional band was produced on the corresponding F1 CA Immunoblot for which the same F1 CA preparation was used, although non-specific background signals were induced on the strips of both immunoblot assays by all four sera. These non-specific reactions could be the reason for the high absorption values in the ELISA. Nevertheless, it can be concluded that anti-YOP antibodies did not influence the specificity of the ELISA or the F1 CA immunoblot, as no yersiniosis patient sera and none of 39 anti-YOP antibody positive sera of healthy blood donors produced positive signals. Taking into account the negligible seroprevalence of plague in Germany and most other European countries, the specificity of 96·1% – which would be sufficient in a population at risk [4] – is inadequate. Serological studies under these conditions would result in very poor predictive values. Therefore, we combined the ELISA with an immunoblot. This procedure is easy to perform and showed 100% specificity in the population studied here probably due to the easy interpretation (yes/no) of a single F1 CA specific band on the nitrocellulose membrane strip (Table 1). The immunoblot assay can be applied as a confirmation test whereas the F1 CA ELISA is suitable for screening of large sample numbers. Both assays correctly identified the plague patient sera but the intensity of the signals in the two assays could not be correlated. This discrepancy might be caused by the different techniques used. For the immunoblot the F1 CA was electrophoresed under denaturing and reducing conditions, so that distinct epitopes may have been altered. However, in the ELISA, the F1 CA was coated onto the surface under mild conditions so epitope structures are less likely to have been affected.

Only 22 out of 26 serum samples of vaccinees contained antibodies reacting to the F1 CA coating (Table 1). This finding accords with the data of different vaccine efficacy studies, which show that only 88–92% of vaccinees produced a measurable anti-F1 CA response [20]. Again two ELISA positive sera out of this group were negative when investigated by F1 CA immunoblot (Table 2). These sera did not contain specific antibodies associated with yersiniosis. A false positive reaction could have arisen by the mechanisms described above. Little is known about the immune response to *Y. pestis* antigens in naturally occuring bubonic plague in humans. In a mouse model of pneumonic plague it has been shown that F1 CA is the major immunodominant antigen to which all infected animals reacted even if treated early with antibiotics. Starting treatment early reduced or suppressed the immune response to YOPs. These findings in animals can explain the failure to detect anti-YOP antibodies in Malagsy plague patients as all

were treated on clinical diagnosis (Table 1). Anti-YOP antibodies from preceeding infection with *Y. enterocolitica* or *Y. pseudotuberculosis* would not be expected as Madagascar is believed to be a non-endemic area for both agents (probable explanation in Madagascar). Assays applying F1 CA give the highest sensitivity of all tests used for serodiagnosis.

However, two facts should be considered when using diagnostic assays based on F1 CA. Firstly, a significant proportion of the population seems to be unable to produce a detectable anti-F1 CA response, as observed during a recent plague outbreak on Madagascar. Only 82·1% of the bacteriologically confirmed cases had anti-F1 CA-specific antibodies. Secondly, in the first few days of plague infection, only F1 CA is present in the sera of patients and anti-F1 CA antibodies do not develop until later [7, 13, 14]. During this early period, only rapid assays detecting the presence of F1 CA [13] or the plague bacilli by PCR [21] would be of any value.

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