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Validation of Serological Testing for Anti-*Treponema pallidum* from Postmortem Blood on the Siemens-BEP®-III Automatic System

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Keywords
Infectious disease serology · Postmortem blood · Blood testing · Tissue donation · *Treponema pallidum*

Summary
Background: Infectious disease marker testing is obligatory for the release of human tissue for transplantation. Most CE-marked tests are not validated for postmortem blood. In a previous study we have validated the testing for anti-HIV-1/2, anti-HCV, HBsAg, and anti-HBc. Here, we present the validation of testing for antibodies against *T. pallidum*, which is the last marker obligatory for tissue release for transplantation. **Methods:** 17 samples of postmortem sera and 10 samples of both pre- und postmortem sera were obtained from cornea donors and tested for anti-*T. pallidum* on the Siemens-BEP-III-System. These sera were spiked with anti-*T. pallidum*-positive standard sera in concentrations which give low- and high-positive results at the respective dilution. **Results:** Two of the unspiked postmortem sera were false-positive most likely due to intense hemolysis (free hemoglobin > 50 mg/dl). Of the 25 negative postmortem sera, none of the spiked samples was false-negative after 0, 24 and 60 h. **Conclusion:** There is no indication that postmortem samples give false-negative or false-positive results with the test system and test kits used in cases of low hemolysis. The procedure described might serve as a model for validating other test kits on postmortem samples.

Introduction

Serological testing for anti-HIV1/2, HBsAg, anti-HBc, anti-HCV and anti-*T. pallidum* is mandatory for the viral safety of tissue donations in Germany (TPGGewV of March 26, 2008) [1] and the EU directives 2004/23/EC and 2006/17/EC [2, 3]). In accordance with EU directive 2006/17, postmortem blood may be used, if premortem samples are not available, but must be taken not later than 24 h postmortem. However, most CE-marked infectious disease test equipment is not validated for testing postmortem blood. Therefore, the Paul-Ehrlich-Institut (PEI), the German authority responsible for tissue preparations, recommends the validation of all test systems used for serological investigation of postmortem blood samples. In a previous study we successfully validated the serological testing for anti-HIV-1/2, anti-HCV, HBsAg ,and anti-HBc from postmortem blood on the Siemens-BEP-III System [4]. Here we present the validation of testing for antibodies against *T. pallidum*, which is the last marker obligatory for tissue release for transplantation.

Material and Methods

Blood Samples

From February to April 2010, postmortem blood samples were collected from 23 (no. 1–23 in the tables) and pre- and postmortem samples from another 10 cornea donors (nos. 24–33 in the tables) of the University Tissue Bank of the Charité, Cornea Bank Berlin, Campus Virchow-Clinic, Germany. Consent for tissue removal and serological tests were obtained in advance under the provisions of the German Transplantation Law (TPG). Age and sex of the donors as well as the time post mortem of sampling were documented, starting with serum no. 7 (table 3 to table 7). Serum no. 1–6 were only used for a pilot study (table 2). Immediately after standardized blood sampling (skin disinfection, puncture of the sub-

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Table 1. OD_{cutoff}/OD_{sample} of 2 anti-*T. pallidum*-positive standard sera and one blood donor serum diluted with various concentrations of negative sera in order to establish suitable spiking dilution[a]

Anti-*T. pallidum*-positive standard, dilution	Standard I		Standard II		Anti-*T. palladium* highly reactive blood donor sample	
	negative test kit control	negative blood donor serum	negative test kit control	negative blood donor serum	negative test kit control	negative blood donor serum
1:5	*7.364*	*7.422*	2.513	2.896	*9.896*	*10.326*
1:25	*2.730*	*2.669*	1.132	1.210	*3.253*	*3.322*
1:125	*1.159*	*1.057*	0.729	0.789	*1.131*	*1.235*
1:625	0.764	0.684	0.644	0.648	0.790	0.712
1:3,150	0.628	0.637	0.662	0.531	0.677	0.607
1:6,250	0.642	0.627	0.696	0.571	0.633	0.589
1:12,500	0.636	0.629	0.585	0.568	0.647	0.596

[a]OD_{cutoff}/OD_{sample}: reactive ≥ 1.000; indeterminate: 0.999–0.856; negative <0.856.
Negative sera were weakly reactive when spiked with a dilution of 1:125 of Standard I or 1:25 of Standard II.

clavian vein with sterile equipment, obtaining at least 6 ml postmortem whole blood), the samples were centrifuged at 3,000 × *g* for 15 min (Heraeus Multifuge 3 L-R, Langenselbold, Germany). The serum was divided in three portions and frozen at 30 ± 5 °C. 12–16 h before testing, the samples were transferred to a refrigerator with +2 to +8 °C.

Spiking of Postmortem Serum Samples

For spiking of postmortem serum samples, we used two different anti-*T. pallidum*-positive standards of the National Institute for Biological Standards and Control, Blanche Lane, South Mimms, Potters Bar, Hertfordshire, EN6 3QK, United Kingdom (NIBSC; *www.nibsc.ac.uk/about_us.aspx*):

- *Standard I:* NIBSC Code 05/132 (WHO International Standard 1 st IS for human syphilitic plasma IgG and IgM) (for further details see: *www.nibsc.ac.uk/documents/ifu/10-B590.pdf*),
- Standard II: NIBSC Code 10/B590 (Anti-Syphilis Quality Control Reagent Sample 2) (for further details see: *www.nibsc.ac.uk/documents/ifu/05–132.pdf*).

The PEI is currently not able to provide reference material for anti-*T. pallidum* testing. We wanted to spike the postmortem sera with a quantity of positive serum to give a low-positive and a high-positive test result. In order to find out the appropriate volumes for spiking, serial dilutions were made with a negatively tested blood donor serum and the negative control of the test kit and both positive standards, altogether 4 series. The serial dilution factor was 5, beginning with a 1:5 dilution and up to a dilution of 1:3,150. For the end of the series, the dilution factor was 2 (1:6,250 and 1:12,500). The samples were measured immediately after spiking and, after 24- and 60-hour storage at 4 °C. In order to confirm the correct dilution, a dilution series was tested with the same dilutions of a positive donor sample with a high titer of *T. pallidum*-specific antibodies.

We then tested 6 postmortem sera spiked with both standards to fine-tune the dilutions. High and low spiking was done in two steps to obtain the correct dilution.

Finally, we spiked the 17 postmortem samples of donors with postmortem material only and 8 out of the 10 samples of donors with pre- and postmortem samples. As the quantity of reference material Standard I was not sufficient for high and low spiking, only low spiking (corresponding to high dilution) was performed.

The first aliquot of the postmortem sample was tested unspiked as a negative control, the second aliquot was spiked with a high dilution of antibody, with expected results near the cut-off value (low-spiked), and the third aliquot was spiked with a high concentration of the antibody (high-spiked). The spiking was performed using a calibrated Biomaster® Eppendorf pipette. The freeze-dried Standard I was dissolved in a sodium chloride solution (1 ml isotonic sodium chloride solution 0.9%, Braun, Melsungen, Germany), the Standard II was delivered in a vial of 4 ml ready for use.

Test Kits

The following test kit was used: SIEMENS Enzygnost® Syphilis (Siemens Healthcare Diagnostics Products GmbH, Marburg; Germany). SIEMENS Enzygnost Syphilis is a competitive one-step enzyme immunoassay. *T. pallidum*-specific antibodies contained in the sample and the peroxidase(POD)-labeled antibodies (Anti-*T. pallidum* / POD conjugate) compete for binding to the *T. pallidum* antigens coated onto the wells of the microtitration plate [5]. The intensity of the color produced is inversely proportional to the activity of *T. pallidum*-specific antibodies contained in the sample. All measurements were performed in the Laboratory for Infectious Diseases, Serology of the Charité – University Medicine Berlin, Institute of Transfusion Medicine, Campus Charité Mitte, on the BEP III System (Siemens).

Presentation of Results and Statistics

In order to make results of different test runs (with different cutoffs) comparable, they are given as OD_{cutoff}/OD_{sample} ratio. Numbers equal to or greater than 1 represent positive results.

Results

Tissue Donors

All premortem samples obtained up to 7 days before death were unequivocally negative in all tests. The postmortem blood samples no. 7–33 were taken from 12 female and 15 male donors at the time of cornea removal. The median age of the donors was 67 years (48–78 years). The postmortem blood samples were collected between 11 and 58 h postmortem.

Determination of Standard Dilutions for Spiking Post Mortem Sera

For Standard I, a dilution of 1:125 and for Standard II a dilution of 1:25 was found to still give weakly positive results (table 1) and thus chosen as standard low spiking dilution for a pilot study with 6 postmortem sera. The high spiking dilution was arbitrarily set at 1:10.

When postmortem sera were tested (table 2), we found one sample false-negative (no. 1) and one sample false-equivocal (no. 3) for Standard I at a dilution of 1:125. For Standard II in a dilution of 1:25, we found one sample false-equivocal (no. 6) (table 2). Therefore, we performed the further low-spiking procedures for Standard I in a dilution of 1:60 and for Standard II in a dilution of 1:20 for the low (adapted low in the tables) and 1:10 for the high spiking.

Table 2. OD_{cutoff}/OD_{sample} of 6 postmortem sera spiked with two different concentrations of either anti-*T. pallidum* positive Standard I or Standard II[a]

No.	Standard I		Standard II	
	standard low (1:125)*	standard high (1:10)*	standard low (1:25)*	standard high (1:10)*
1	*0.812*	5.147	1.122	1.878
2	1.115	4.944	1.083	1.870
3	*0.874*	4.861	1.103	1.927
4	1.090	5.087	1.037	1.768
5	1.066	4.781	1.103	1.866
6	1.034	4.888	*0.986*	1.902

[a]$OD_{cut\ off}/OD_{sample}$: reactive ≥1.000; indeterminate 0.999–0.857; negative <0.857.
The higher standard dilutions used to generate samples with low amounts of *T. pallidum* antibodies result in concentrations insufficient to guarantee positivity for each serum. It was adapted to 1:60 for Standard I and 1:20 for Standard II.

Testing of 17 Tissue Donors with Only Postmortem Samples

All 17 spiked samples were tested positive directly after spiking. In all 17 samples low-spiked with Standard I and II the cutoff-to-sample ratio was shifted slightly towards negative during the first 24 h (tables 3, 4). An exception is sample 13 spiked with adapted low concentration of Standard II, which was indeterminate immediately after spiking. Results were, however, comparable to those of the other sera after 24 and 60 h. Unfortunately, the quantity of this serum was not sufficient to repeat the spiking. For most samples, the cutoff-to-sample ratio rose again when tested 60 h after spiking.

Testing of 10 Tissue Donors with Pre- and Postmortem Samples

Ten samples were tested of the 10 tissue donors of whom pre- as well as postmortem samples were available (table 5). All premortem samples were unequivocally negative. Two of the postmortem samples were false-positive. Both samples (no. 32 and 33) were visibly hemolytic. Free hemoglobin was 50 or 95 mg/dl. These two samples were excluded from spiking. Two other samples were visibly hemolytic, and the results shifted in the direction of positive, but as they were still clearly negative, they were not excluded from further analysis. For the remaining 6 non-hemolytic sera there was no substantial difference between the pre- and postmortem cutoff-to-sample ratio.

Table 3. OD_{cutoff}/OD_{sample} of postmortem samples spiked with adapted low concentration of Standard I anti-*T. pallidum*-positive standard measured immediately and 24 and 60 h after spiking

Donor characteristics				Standard I, spiking concentration 1:60 (adapted low)				
No.	age, years	Sex	PM time, h	0 h	24 h	difference 0/24 h, %	60 h	difference 24/60 h, %
7	56	m	13	1.730	1.458	–15.7	1.576	8.1
8	66	m	54	1.730	1.411	–18.4	1.564	10.8
9	57	w	24	1.803	1.427	–20.9	1.602	12.3
10	48	m	41	1.879	1.494	–20.5	1.525	2.1
11	78	w	17	1.800	1.421	–21.1	1.557	9.6
12	66	w	53	1.781	1.477	–17.1	1.595	8.0
13	60	m	51	1.766	1.468	–16.9	1.643	11.9
14	60	w	44	1.603	1.431	–10.7	1.555	8.7
15	75	m	25	1.785	1.505	–15.7	1.638	8.8
16	73	w	32	1.773	1.437	–19.0	1.514	5.4
17	56	m	50	1.781	1.470	–17.5	1.560	6.1
18	72	m	25	1.676	1.400	–16.5	1.448	3.4
19	73	w	41	1.908	1.560	–18.2	1.474	–5.5
20	75	m	34	1.796	1.521	–15.3	1.528	0.5
21	58	m	24	1.716	1.421	–17.2	1.539	8.3
22	73	m	31	1.628	1.400	–14.0	1.669	19.2
23	61	m	12	1.669	1.425	–14.6	1.474	3.4
Mean						–17.2		7.4
Standard deviation						2.56		5.34
Median				1.78	1.45	–18.5	1.56	7.1

PM time = Time in hours between death and drawing of blood sample.
[a]$OD_{cut\ off}/OD_{sample}$: reactive ≥1.000; indeterminate 0.999-0.857; negative <0.857.

Table 4. OD_{cutoff}/OD_{sample} of postmortem samples spiked with adapted low and high concentration of Standard II anti-*T. pallidum*-positive standard measured immediately and 24 and 60 h after spiking[a]

Donor characteristics				Standard I, spiking concentration 1:60 (adapted low)					Standard I, spiking concentration 1:10 (adapted high)				
No.	age, years	Sex	PM time, h	0 h	24 h	difference 0/24 h, %	60 h	difference 24/60 h, %	0 h	24 h	difference 0/24 h, %	60 h	difference 24/60 h, %
7	56	m	13	1.213	1.042	–14.1	1.037	–0.5	1.842	1.628	–11,6	1.732	6,4
8	66	m	54	1.290	1.135	–12.0	1.145	0.9	1.773	1.724	–2,8	1.679	–2,6
9	57	w	24	1.281	1.120	–12.6	1.191	6.3	1.965	1.684	–14,3	1.612	–4,3
10	48	m	41	1.312	1.131	–13.8	1.168	3.3	1.904	1.67	–12,3	1.829	9,5
11	78	w	17	1.394	1.212	–13.1	1.179	–2.7	1.979	1.733	–12,4	1.682	–2,9
12	66	w	53	1.294	1.140	–11.9	1.234	8.2	1.984	1.748	–11,9	1.770	1,3
13	60	m	51	0.911**	1.169	*28,3*	1.220	4.4	1.952	1.766	–9,5	1.729	–2,1
14	60	w	44	1.222	1.108	–9.3	1.203	8.6	1.871	1.727	–7,7	1.696	–1,8
15	75	m	25	1.273	1.174	–7.8	1.207	2.8	1.917	1.742	–9,1	1.826	4,8
16	73	w	32	1.331	1.158	–13.0	1.292	11.6	1.867	1.727	–7,5	1.715	–0,7
17	56	m	50	1.256	1.158	–7.8	1.317	13.7	1.921	1.67	–13,1	1.699	1,7
18	72	m	25	1.287	1.102	–14.4	1.260	14.3	1.785	1.638	–8,2	1.625	–0,8
19	73	w	41	1.692	1.295	–23.5	1.328	2.5	2.299	1.887	–17,9	1.819	–3,6
20	75	m	34	1.306	1.199	–8.2	1.255	4.7	1.93	1.628	–15,6	1.710	5,0
21	58	m	24	1.458	1.190	–18.4%	1.291	8.5	2.007	1.687	–15,9	1.735	2,8
22	73	m	31	1.306	1.126	–13.8	1.287	14.3	1.803	1.544	–14,4	1.602	3,8
23	61	m	12	1.234	1.081	–12.4	1.162	7.5	1.947	1.617	–16,9	1.747	8,0
Mean						–12,8		6.4			–11.5	1.0	1.0
Standard deviation						2,6		5.3			3.9		4.1
Median				1,29	1,14	–11,6	1.22	7.01	1.92	1.69	–12.2	1.72	1.7

PM time = Time in hours between death and drawing of blood sample.
[a]$OD_{cut\ off}/OD_{sample}$: reactive ≥ 1.000; indeterminate 0.999-0.857; negative <0.857
**Outlier: not considered for 'median' and 'difference 0/24 h'.

Table 5. OD_{cutoff}/OD_{sample} of unspiked pre- and postmortem samples of the same tissue donors

No.	Age, years	Sex	PM time, h	Premortem	Postmortem
24	61	w	15	0.475	0.469
25	73	w	11	0.472	0.471
26	70	w	48	0.463	0.473
27	67	w	58	0.468	0.441
28	71	w	10	0.454	0.453
29	78	m	27	0.463	0.453
30	77	m	12	0.463	0.598**
31	74	m	35	0.472	0.607**
32	61	m	26	0.457	*1.025**
33	70	w	48	0.466	*2.734**

PM time = Time in hours between death and drawing of blood sample.
$OD_{cut\ off}/OD_{sample}$: reactive ≥1.000; indeterminate 0.999–0.857; negative <0.857.
*Distinct hemolysis (free hemoglobin > 50 mg/dl).
**Visible hemolysis (free hemoglobin not measured as results were still positive).

8 pre- and 8 postmortem samples were spiked with two different concentrations of Standard I and Standard II and tested 3 times within 60 h (tables 6, 7). Both the low- and high-spiked samples showed correct positive results for anti-*T. pallidum*. False-negative results were not observed (sample 27 low-spiked with Standard I was indeterminate when tested immediately after spiking, but positive in the two subsequent tests). Furthermore, no differences depending on the postmortem blood collection time, age, or sex could be demonstrated. The optical density of the samples did not change noticeably with increasing postmortem removal time (tables 6, 7). Differently from the results of the 17 spiked postmortem samples, there was no substantial difference when OD at 0, 24, and 60 h were compared.

Discussion

Serological testing of tissue donors for antibodies against *T. pallidum* in tissue donations in Germany is mandatory. The bacterium is transmissible by blood, and even if published transmissions by blood have become extremely rare, the incidence of syphilis in Germany is rising. This increase can be largely attributed to the group of men who have sex with men (MSM) [6]. In many tissues for transplantation, virus inactivation methods further reduce the

Table 6. OD_{cutoff}/OD_{sample} of spiked (adapted low 1:60, standard high 1:10) pre- and postmortem samples of 8 tissue donors with Standard I[a]

No.	PM time, h	0 h				24 h				60 h			
		low		high		low		high		low		high	
		PRM	PM	PRM	PM	PRM	PM	PRM	PM	PRM	PM	PRM	PM
24	15	1.438	1.391	4.127	3.560	1.405	1.311	4.165	3.898	1,460	1,450	4,253	4,020
25	11	1.306	1.666	3.980	4.044	1.457	1.693	4.182	4.415	1,446	1,888	4,100	5,151
26	48	1.527	1.459	4.339	3.965	1.539	1.445	4.116	3.812	1,432	1,460	3,522	4,362
27	58	1.387	0.981	3.934	3.830	1.304	1.208	3.958	3.988	1,370	1,248	3,883	4,100
28	10	1.438	1.408	3.919	3.717	1.294	1.258	3.798	3.676	1,370	1,420	3,853	3,714
29	27	1.424	1.364	4.044	3.934	1.306	1.266	3.913	3.973	1,475	1,309	4,004	3,897
30	12	1.563	1.474	3.980	4.195	1.405	1.407	3.913	3.913	1,556	1,426	4,218	3,973
31	35	1.414	1.404	3.859	3.874	1.402	1.390	3.794	3.958	1,478	1,336	4,133	3,635

PM time = Time in hours between death and drawing of blood sample; PRM = premortem; PM = postmortem.
$OD_{cut\ off}/OD_{sample}$: reactive ≥1.000; indeterminate 0.999–0.857; negative <0.857.

Table 7. OD_{cutoff}/OD_{sample} of spiked (adapted low 1:20, standard high 1:10) pre- and postmortem samples of 8 tissue donors with Standard II

No.	PM time, h	0 h				24 h				60 h			
		low		high		low		high		low		high	
		PRM	PM	PRM	PM	PRM	PM	PRM	PM	PRM	PM	PRM	PM
24	15	1.135	1.127	1.536	1.585	1.162	1.143	1.702	1.739	1,174	1,184	1,350	1,711
25	11	1.140	1.432	1.567	1.818	1.257	1.497	1.825	2.100	1,296	1,726	1,767	2,149
26	48	1.280	1.217	1.872	2.357	1.461	1.249	1.956	2.465	1,219	1,428	1,801	2,595
27	58	1.163	1.220	1.663	1.605	1.179	1.239	1.892	1.784	1,167	1,212	1,780	1,723
28	10	1.272	1.198	1.657	1.647	1.215	1.126	1.766	1.650	1,163	1,209	1,761	1,726
29	27	1.165	1.195	1.587	1.666	1.196	1.126	1.710	1.742	1,158	1,192	1,686	1,686
30	12	1.218	1.228	1.652	1.666	1.175	1.181	1.775	1.699	1,341	1,296	1,786	1,827
31	35	1.157	1.218	1.577	1.679	1.001	1.154	1.847	1.778	1,165	1,200	1,718	1,990

PM time = Time in hours between death and drawing of blood sample; PRM = premortem; PM = postmortem.
$OD_{cut\ off}/OD_{sample}$: reactive ≥1.000; indeterminate 0.999–0.857; negative <0.857.

chance of transmission [7]. Active or past syphilis, however, is also an indication of a more sexually active lifestyle and hence of a greater likelihood of other sexually transmissible infections.

Screening for antibodies against *T. pallidum* is performed by hemagglutination (TPHA) or particle agglutination (TPPA) testing or, more recently, by enzyme immunoassay (EIA). EIA reading can be more easily automated, and no dilution series are necessary for positive samples. They are licensed. Comparability to particle agglutination assays is generally accepted [8, 9], but mistrusted in some recent publications [10].

After death, many changes take place in the blood. Antibodies are not as easily degraded as is e.g. HCV RNA [11]. Nevertheless, false-negative results in infectious disease testing are possible due to inhibiting substances, and false-positive or false-negative results due to autolysis, hemolysis or bacterial growth. Most CE-marked test systems are not validated for testing postmortem blood.

In a previous study on serological testing for HIV, HBV, and HCV with blood samples taken up to 48 h after death, no false-negative results occurred at later time intervals and indicated a high stability of antibodies and antigens in the postmortem process [12]. We could not test for autolysis of antibodies against *T. pallidum* after death because none of the donors was antibody-positive from start nor was serial sampling in dead donors possible. As a surrogate, we obtained pairs of pre- and postmortem sera of 10 cornea donors. The negative premortem sera served as control that the donor was indeed not infected. Any positive result in postmortem serum would then be a false-positive result. We found 2 out of 10 false-positive results due to severe hemolysis (free hemoglobin > 50 mg/dl). Severely hemolytic sera are thus unsuitable for testing.

False negativity may be due to inhibiting substances. To test for inhibiting substances, we spiked postmortem sera with antibody-positive standard sera in dilutions that give weakly positive results in blood donor sera. Weakly positive results were also obtained when negative postmortem sera were spiked. A small inhibiting effect may be assumed as optical densities of negative sera were shifted in the direction of neg-

ative after 24 h (tables 3, 4). However, there was no detectable further shift after 60 h. On the contrary, most signals were even somewhat stronger at 60 h compared to 24 h (tables 3, 4), and thus the postulated inhibiting effect would be instable. Strangely, this effect could not be observed for another group of sera (tables 6, 7). All sera remained positive or at least indeterminate.

The concentrations of the spiking materials from NIBSC were suitable to fulfill the requirements of our validation. From the perspective of the authors, this validation should be extended to include spiked samples from living donors in order to detect any differences between pre- and postmortem blood.

In our opinion, the methodology described in the present study may be used as guidance for validation of serological testing for syphilis in postmortem blood samples.

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Disclosure Statement

The authors declared no conflict of interest.

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