Diagnosing toxigenic *C. difficile*: new confidence bounds show culturing increases sensitivity of toxin A/B EIA, and refute gold standards

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This is an extended version of Mattner et al. (2009) (1)

Abstract

Objective: To scrutinize published sensitivity estimates obtained using questionable gold standards by comparing sensitivities of culturing *C.difficile* in commercially available media followed by enzyme immuno assay (EIA) toxin A or B detection (culture test) with applying the EIA to stool samples alone (direct test).

Methods: In 2008, consecutive stool samples were cultured on *C. difficile* selective culture media (medium I: CDSA (Becton Dickinson), medium II: CLO-agar (BioMérieux), medium III: *C. difficile*-agar according to Brazier (Oxoid)). Additionally, a direct test was performed (Ridascreen, r-biopharm), which was also used to confirm toxin A - or B-production of cultured *C. difficile*. New confidence bounds for sensitivities were applied, without assuming any perfect reference test or any conditional independence of the tests compared.

Results: Of 256 liquid stool samples, 18.4% were diagnosed as positive by at least one of the four tests, 12.8% with culture medium I, 16.4% with II, and 13.6% with III, and 10.1% by the direct test. Assuming culture tests to be at least as specific as the direct test yields an upper bound of 61% (upper 95% confidence bound (CB) 81%) for the sensitivity of the direct test. Assuming a prevalence of 15% yields sensitivity gains of the culture tests of at least 18% (lower 95%CB -4%) for medium I, 40% (95% CB 21%) for II, and 23% for III (95% CB 2%).

Conclusion: Published high sensitivities of direct toxin A/B EIAs, up to 96%, and the correctness of the cytotoxicity test assumed for their estimation, are doubtful. With culture medium II sensitivity gains of at least about 20% are obtainable. Direct toxin A/B EIAs alone are insufficiently sensitive for the clinical diagnosis of *Clostridium difficile* infections.

Introduction

Increasing rates of *C. difficile* infections (CDI) in many different countries demand effective infection control strategies. A sensitive and rapid laboratory diagnostic is an important condition for the initiation of specific infection control measures (2-6). The aim of a microbiological diagnostic is the proof of the toxigenic potential for the production of toxin A or B of *C. difficile* (toxigenic *C. difficile*). If toxigenic *C. difficile* is diagnosed, then targeted antibiotic treatment should be initiated to prevent severe clinical courses and specific infection control measures started to prevent future nosocomial transmission. *C. difficile* toxin A and B EIAs (CdT) for stool samples –in the following called direct test- and selective culture media are commercially available (7, 8). The cytotoxicity test is regarded as a reliable reference test (9) but it is time consuming to be performed in routine diagnostic laboratories and not all laboratories perform cell cultures.

Centers for Disease Control and Prevention (CDC) and European Centre for Disease Prevention and Control (ECDC) recommend a combination of tests such as toxin A/ B-EIA or GDH-EIA combined with a culture medium (10-12). Nonetheless, often only rapid tests are performed (13, 14). No recommendations were made which kind of culture media should ideally be used. Therefore we compared for each of three commercially available culture media (without blood, with sheep blood, and with horse blood), the results of applying toxin A/B EIA to any *C difficile* cultures grown on the given medium with the results obtained by applying toxin A/B EIA directly to the respective stool samples.

In addition, we discuss recently published sensitivities and specificities for toxin A and B EIA – reported between 67 – 92% and 90 – 98%, respectively (9) - focussing on the reference methods used.

We consider the present comparison of toxin A/B EIA with and without culturing not only as a model for similar investigations, but also as a particularly important example, the latter since the direct toxin A/B EIA test is very common for diagnosing toxigenic *C. difficile* due to its convenience but apparently not due to any well-founded evidence for its accuracy.

Alternative microbiological test systems with possibly higher accuracies for diagnosing

toxigenic *C.difficile* in stool samples, such as culture media favouring spore germination by ethanol shocks (15) or taurocholate supplement to culture medium, or different molecular techniques (e.g. polymerase chain reaction (PCR) , loop-mediated isothermal amplification (LAMP) (16-19)), are not considered here. Instead we emphasize the fallacy of calculating and even publishing wrong high accuracies of diagnostic tests, obtained by uncritically using doubtful reference tests, with the probable consequence of misdiagnosing and mistreating many patients.

Methods

From February to March 2008 all liquid stool samples sent to a university microbiological laboratory were investigated for toxigenic *C. difficile*. Only the 256 first samples from 256 patients were included in our study.

A *C. difficile*-Toxin A and B-EIA was performed on a daily basis directly on stool specimens (CdT-direct-test (Ridascreen, r-biopharm)). In addition, each stool specimen was anaerobically cultured on three different culture media (culture medium I:CDSA, Becton Dickinson (Peptone 32g/l, neutralred 0.03g/l, cycloserine 0.25g/l, cefoxitine 0.016g/l); culture medium II: CLO, BioMérieux (peptone 21g/l, sheep blood 50ml/l, cycloserine 0.1g/l, cefoxitine 0.008g/l, amphotericine B 0.002g/l); culture medium III: *Clostridium difficile* selective agar according to Brazier, Oxoid, (peptone 23 g/l, defibrinated horse blood 10 ml/l, egg york 40 ml/l, p-hydroxyphenylacetate 1.0 mg/l, cycloserine 0.25g/l, cefoxitine 0.008g/l, amphotericine B 0.008 g/l, cholate 1.0 g/l)) for 48h at 37°C. Morphologically suspicious growing colonies were tested for *C. difficile* using an latex-agglutination test for cell wall antigen (Oxoid), fluorescence at 366 nm wavelengths and in the case of positivity for the potential of toxin A or B production using the Toxin A and B-EIA (Ridascreen, r-biopharm) according to the manufacturers recommendations. Each such “culture test” was defined as positive if the Toxin A and B EIA of the tested colonies was positive.

As to date no perfect reference system for the diagnosis of toxigenic *C. difficile* is available, no accuracy values (sensitivity and specificity) can be determined (20). But it is possible to determine an upper bound for the sensitivity of the direct test (point estimate and upper 95% confidence bound) subject only to the following important assumption:

(A) The specificity of diagnosing Cd toxin A or B by EIA with at least one culture medium is at least as high as the specificity of the EIA performed directly on the stool sample.

This assumption is plausible, as in a culture test, the EIA is applied to a part of a culture identified as *C. difficile*, and thus to a material more specific than stool for the diagnosis of interest.

It is further possible to compute minimum sensitivity gains through culturing with each medium (point estimate and lower 95% confidence bound) subject to the following slightly weaker version of the above assumption:

(B) The specificity of diagnosing Cd toxin A or B by EIA on the given culture medium is at least as high as the specificity of the EIA performed directly on the stool sample,

and assuming a prevalence of toxigenic *C. difficile* of 15% in liquid stool samples sent to the laboratory (21, 22), (7% in infants (23)). However, this latter assumption is of minor importance: For a prevalence of x%, point estimates and lower confidence bounds have only to be multiplied by 15 / x. The statistical method used here for computing the bounds just mentioned is explained and justified in detail in the companion paper (24), aimed at mathematical statisticians. Very briefly put, (24) mainly consists of a mathematical analysis of the classical latent class model (25), developing in particular the consequences of the mathematical version of assumption (A) or (B), without assuming any perfect reference test and without imposing the apparently unjustified conditional independence assumption (20). This analysis reduces our problem of finding confidence bounds to a more standard problem solved well in (26). The inevitable overlap of (24) with the present paper consists in presenting there just enough of the present data and their background as are necessary to illustrate there the new confidence bounds, and to enable a reader of the present paper to verify the calculations leading to the numerical results presented here, down to the statistical software (26) used.

To compare our own with published data we performed a Pubmed search for *C. difficile* toxin A/B EIAs. All studies containing accuracy data referring to cytotoxicity tests or toxigenic cultures were included and used to investigate our hypothesis: published accuracy values may not be reliable if it is not known which test should be used as the reference system.

Results

Out of 256 first stool samples of patients with loose stool, 47 were tested positive in at least one of the culture tests or the CdT-direct test (Table 1). 26 samples were positive in the CdT-

direct test. Out of them *C. difficile* could not be cultured in 3 patients, in one case CdT-toxin A and B EIA was negative in the tested colonies. 209 samples were negative in all tests. Toxigenic *C. difficile* culture was positive for culture medium I in 33 (12.8%), II in 42 (16.4%) and III in 35 (13.6%) patients. In 4 (culture medium I), 7 (II) and 5 (III) patients cultured *C. difficile* were non-toxigenic (= negative CdT-EIA of the colonies). Subject only to assumption (A), the sensitivity of the direct is at most 61% (upper 95% confidence bound (CB) 81%). Subject to assuming (B) and a prevalence of 15%, minimum sensitivity gains of culture tests compared to the CdT-direct test were 18% (-4% lower 95% CB minimum sensitivity gain not significant) for culture medium I, 40% (21% lower 95% CB) for II and 23% (2% lower 95% CB) for III. For comparison to widely published accuracy value calculations (Table 2) in particular in (7), the sensitivity of the CdT direct test would be calculated as 51% (upper 95% confidence bound 64%) if the reference was defined as “at least one culture test positive“.

Discussion

C. difficile strains (e.g. ribotype 027) leading to nosocomial outbreaks and severe clinical courses emerged worldwide over the last few years (2-6). To start a specific antibiotic treatment und initiate infection control measures for the prevention of nosocomial transmission a sensitive and fast microbiological diagnostic of toxigenic *C. difficile* is needed. In many laboratories only fast direct tests (toxin A/B EIA or GDH-EIA) are routinely performed (13, 14). To date only few accuracy data of different tests for detection of toxigenic *C. difficile* is available. Therefore, we performed a study to determine the sensitivity gain of three commercially available culture media in comparison to a CdT direct test. Surprisingly the CdT-direct test (Ridascreen, r-biopharm) has a sensitivity of at most 81% (upper 95 % CB) and was, assuming 15% prevalence, at least 21% (lower 95 % CB) less sensitive than culture medium II (CLO-Agar, BioMérieux with confirmation of toxigenecity by CdT-EIA). The sensitivity gain was maximal for culture media II followed by III. Culture medium I (claimed to be more sensitive than CCFA, which is recommended by reference laboratories) was not significantly different from the CdT-direct test. Even though our study was limited by the fact that the confirmation of toxinogenicity was performed using a toxin A/B EIA and not by using the cytotoxicity test, published high sensitivity values as in (7) and in further studies reported in Table 2 were questioned by our results and are thus believed to overestimate the true sensitivity. This discrepancy might be due to different reference systems chosen. E.g. the reference system chosen for most publications was the cytotoxicity test and not a sensitive culture test. Other studies refer to a less sensitive culture medium such as CCFA-agar (16) which showed no sensitivity gain in our test system either. A more comprehensive study tested against CCEYL agar similar to medium III in our study (9). In our study a cytotoxicity test was not available. But other authors already showed that the cytotoxicity test might be less sensitive compared to several culture media or PCR (27-30). When CdT direct tests were tested against sensitive toxigenic *C. difficile*-culture media, also surprisingly low sensitivity values were obtained (31-35). On the other hand, a more recent publication found a low (61%) sensitivity of a direct test compared to the cytotoxicity neutralization test (36).

Even if culture methods were used as “gold standard” they may differ by the plate culture method used, as our results suggest.

Furthermore, culture enrichment techniques –not investigated in our study- are also plausible to increase *C.difficle* detection (37), but a sensitivity gain of an enrichment culture compared to a plate culture will depend on both the medium culture and the enrichment method used. It could well happen that a sensitivity gain of an enrichment method could be counterbalanced by the choice of a lowly sensitive culture agar plate.

Other recent publications evaluate and claim accuracy values comparing more than two different tests (e.g. glutamate dehydrogenase (GDH)-test, Toxin A/B EIA, cytotoxicity test and PCR) also including diagnostic algorithms (16, 18). In these attempts at improving the diagnosis of toxigenic *C. difficile*, all studies fail to justify the gold standard used for their accuracy value calculations. One study assumed toxigenic *C. difficile* as true if four tests (GDH-test, toxin A/B EIA, cytotoxicity neutralization test and PCR) were positive, and this even without performing a culture (16). A more reliable study claimed a still higher sensitivity of a LAMP test for the detection of the tcdA-gene compared to culture method (CCFA-agar, which appeared to be the least sensitive in our study, however). Those patients whose samples were detected as positive in the LAMP test and negative in the culture developed CDI later on, thus proving the initial result of the LAMP test (17). The studies shown in Table 2 and those discussed above calculated accuracy values relying on different reference systems.

Hence, the important question arises:

How can accuracy values be obtained for toxigenic C. difficile tests if it is not clear which reference method is appropriate? Which sensitivity values should we believe and use as a basis for deciding which test to introduce in a laboratory for routine use?

We partially answered these questions by bounding from above the sensitivity of a direct test and, with an exemplary prevalence assumption, by calculating lower bounds for sensitivity gains of culture tests.

Under the plausible assumption that a culture test is at least as specific as a CdT-direct test, our confidence bounds are statistically correct and practically not improvable, as we show in (24) by using the latent class models (25) and confidence bounds for differences of multinomial parameters (26).

We believe that our statistical method is useful also in other cases and that it should replace unreliable and potentially misleading “calculations of sensitivities” (e.g. those reported in table 2).

For the clinical use our results suggest that a toxin A/B EIA applied as a direct test alone is not reliable due to its low sensitivity. A second test increasing the sensitivity should be used. As toxigenic culture or cytotoxicity test as time consuming methods are leading to a diagnostic delay, it will be interesting whether in future molecular methods could fill the gap. For the comparison of a direct molecular test with a *C. difficile* culturing confirmed by the same molecular test for toxin A/B genes our statistical method could be used for accuracy comparisons not relying on any doubtful reference system.

Note

Fa. Oxoid supplied culture medium III and the latex agglutination test. The study was core funded. The authors have no conflicts of interest.

Authors contributions:

Frauke Mattner planned the study, interpreted data, and wrote the manuscript.

Ingo Winterfeld chose different culture media, performed all laboratory tests, and was responsible for data structure and management.

Lutz Mattner interpreted data, developed a statistical method, applied it to the data, and was involved in writing the manuscript.

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Table 1: Results of 256 consecutive first stool samples of patients with diarrhoea: *C. difficile* toxin A+B test performed from stool, toxin A or B producing "positive" culture media

Number of investigated stool samples (n=256, out of them 47 positive in at least one test)	Toxigenic culture			CdT-direct test from stool samples
	I	II	III	
18	+	+	+	+
14	+	+	+	-
1	+	+	-	-
1	-	+	+	-
1	-	-	+	-
4	-	+	-	-
1	-	+	+	+
3	-	+	-	+
4	-	-	-	+
Sum of positive results	33	42	35	26

Toxigenic culture I: CDSA, BD
Toxigenic culture II: CLO, BioMérieux
Toxigenic culture III: *Clostridium- s*elektive agar according to Brazier, Oxoid
CdT-direct test performed with stool samples: Ridascreen, r-Biopharm

Table 2: Reported sensitivities and specificities of different *C. difficile* toxin A/B EIAs (*C. difficile* toxin A/B EIA in stool samples or toxin A/B producing *C. difficile* („toxigenic“ culture)). For the determination of accuracy values different reference systems had been used in below noted publications. As no optimal reference system exists, we call published sensitivities and specificities “reported“ sensitivities and specificities

Authors and investigated diagnostic tests	Number of stool samples investigated	Numerators (Reference tests) used for accuracy value calculation	Reported sensitivity	Reported specificity	Notes
Van den Berg et al.(19),					
Meridian, ICTAB, Biosience	367 samples of 300	Cytotoxicity test	91%	97%	
Europe, Boxtel, The Netherlands	patients	toxigenic culture	79%[a]	99%[a]	Accuracy values recalculated using toxigenic culture as numerator
Rüsmmann et al.(8)	383 samples	Positivity of all 3 tested EIAs			Positivity of all three tested EIAs or toxigenic culture or NAAT in case of discordant results
TechLab Tox A/B II	383 samples		88%	100%	
Ridascreen, r- Biopharm	383 samples		92%	100%	
Oxoid Remel	383 samples		93%	100%	
Diederen et al. (38)		Cytotoxicity test			
Meridian, ICTAB	35 samples of 33 patients		88.6%	Not determinable.	Only samples positive in cytotoxicity test were investigated
Planche et al. (7, 8, 39)					
TechLab Tox A/B II	2158 (6)	Cytotoxicity test with or without toxigenic culture	84%	98%	
Meridian Premier	2891 (9)	Cytotoxicity test with or without toxigenic culture	95%	97%	
TechLab Quick Check	1307 (4)	Cytotoxicity test with or without toxigenic culture	84%	100%	Systematic review using data from different publications. For the comparison of accuracy values for the respective tests the use of different reference systems applied in different studies (cytotoxicity test or toxigenic culture) was ignored as no statistically significant differences were determined by the authors. Samples were pooled from different studies (number of different studies in brackets) and described as “cases”
Oxoid Remel Xpect	520 (2)	Cytotoxicity test with or without toxigenic culture	82%	96%	
Meridian Immunocard	1982 (6)	Cytotoxicity test with or without toxigenic culture	90%	99%	
BioMérieux VIDAS	62 (1)	Cytotoxicity test with or without toxigenic culture	76%	93%	
Eastwood et al. (9)					
Meridian Premier	600 samples	Cytotoxicity test or toxigenic culture	81%	99%	
Ridascreen, r- Biopharm	600 samples	Cytotoxicity test or toxigenic culture	67% 60%	95% 96%	
The Binding site GA Cd-toxinA/B	600 samples	Cytotoxicity test or toxigenic culture	77% 69%	91% 91%	
Oxoid Remel	600 samples	Cytotoxicity test or toxigenic culture	90% 82%	93% 93%	
Vidas Cd Tox A/B	600 samples	Cytotoxicity test or toxigenic culture	90% 80%	97% 97%	
Oxoid Remel Xpect	600 samples	Cytotoxicity test or toxigenic culture	78% 69%	99% 99%	
Techlab Tox A/B Quik Chek	600 samples	Cytotoxicity test or toxigenic culture	91% 74%	92% 99%	Study comparing nine commercially available *C. difficile* toxin detection assays with cytotoxocoty test and toxigenic culture. In addition a commercially available tcB-PCR is tested. CCEYL was used used as culture medium. Isolates were than harvestedin BHI and tested with the cytotoxicity test.
Meridian Immunocard	600 samples	Cytotoxicity test or toxigenic culture	78% 69%	93% 93%	
TechLab Tox A/B II	600 samples	Cytotoxicity test or toxigenic culture	84% 88%	99% 94%	
Nowak-Weekley (35)					

Meridian Bioscience, Inc., Cincinnati, OH	432 samples	Toxigenic culture	58%	95%	CCFA plus enrichment broth used as reference
Alcala et al.(34)					
X/pect	367 samples	Cytotoxicity test with or without toxigenic culture	49%	96%	CLO-Agar from BioMérieux was used as culture medium
Wampole Tox A/B Quick Check	367 samples	Cytotoxicity test with or without toxigenic culture	55%	96%	
ImmunoCard Toxin A+B	367 samples	Cytotoxicity test with or without toxigenic culture	67%	95%	
Miendje Deyi (40)					
Biostar OIA CdTOX AB	100 samples	Cytotoxicity test	87%	99%	
Immunocard Toxins	100 samples	Cytotoxicity test	91%	100%	
Toxin A/B QUIK CHEKTM	100 samples	Cytotoxicity test	96%	100%	
Xpect	100 samples	Cytotoxicity test t	87%	100%	
Present study		In at least one of three culture tests positive for toxigenic *C. difficile*			Consecutive stool samples (only first samples of symptomatic patients):Ridascreen, r- Biopharm and three culture media with confirmation of toxigenicity through Ridascreen
Ridascreen, r- Biopharm	256 patients		**51%**	98%	
CDSA-Agar	256 patients		70%	100%	Modified CCFA agar, Becton Dickinson
CLO-Agar	256 patients		89%	100%	CLO-Agar containing sheep blood, BioMérieux
Clostridium-agar (Brazier)	256 patients		74%	100%	Clostridium-agar (Brazier) containg horse blood, Oxoid