Testing for toxigenic *C. difficile* with Toxin A and B Enzyme Immuno Assay: A new statistical method detects a high sensitivity gain through culturing and questions old gold standards

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

Abstract

Objective: To compare sensitivities of culturing *C.difficile* in commercially available media followed by 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*. A new statistical method was applied.

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 toxin A and B EIAs, and the correctness of the cytotoxicity tests assumed for their estimation, are doubtful. At least with culture medium II sensitivity gains of at least about 20% are plausible. Such relevant results are obtainable with few and plausible statistical assumptions, without relying on questionable “gold standards”.

Introduction

Increasing rates of *C. difficile* infections (CDI) in many different countries demand effective infection control strategies. A sensitive and rapid microbiological diagnostic is an important condition for the initiation of specific infection control measures (2-6). The aim of a micobiological 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 („direct test“) und selective culture media are commercially available (7, 8). The cytotoxicity test is regarded as a reliable reference test but it is held as too difficult and time consuming to be performed in routine diagnostic laboratories.

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 media (9-11). Nonetheless, often only rapid tests are performed (12, 13). No recommendations were made which kind of culture media should ideally be used. Therefore we investigated three different commercially available culture media (without blood, with sheep blood and with horse blood) for sensitivities comparing them with the sensitivity of a toxin A and B EIA.

In addition, we discuss recently published sensitivities for toxin A and B EIA focussing on the reference methods used.

Methods

From February to March 2008 all liquid stool samples sent to a university micobiological laboratory were investigated for toxigenic *C. difficile*. Only the 256 first samples from 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, all stool specimens were 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) 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 (14). Therefore we determined 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.

We further computed 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. 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 and explained in detail in (15) is based on a careful analysis of the classic latent class model (16) and a recent efficient confidence bound from (17).

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). These samples were regarded negative for the calculation of accuracy values. 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 (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 and B EIA or GDH-EIA) are routinely performed (12, 13). 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 and B EIA and not by using the cytotoxicity test, published high sensitivity values like (7) or table 2 were questioned by our results. 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 (18) which showed no sensitivity gain in our test system either. 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 (19-22). If CdT direct tests were tested against sensitive toxigenic *C. difficile*-culture media, also surprisingly low sensitivity values were obtained (23-27). On the other hand, a more recent publication found a low (61%) sensitivity of a direct test compared to the cytotoxicity neutralization test (28). Even if culture methods were used as “gold standard” they may differ by the culture method used, as our results suggest.

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 (15) by using the latent class models (16) and confidence bounds for differences of multinomial parameters (17).

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 of (7)).

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 tests (*C. difficile* Toxin A oder B in stool samples or Toxin A or 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 used for accuracy value calculation	Reported sensitivity	Reported specificity	Notes
Van den Berg et al.(29),					
Meridian, ICTAB, Biosience	367 samples of 300	Cytotoxicity test	91%	97%	
Europe, Boxtel, The Nederlands	Patients	toxigenic culture	79%[a]	99%[a]	Acuracy values recalculated using toxigenic culture as numerator
Diederen et al. (30)		Cytotoxicity test			
Meridian, ICTAB	35 samples of 33 patients		88.6%	n.d.	Only samples positive in cytotoxicity test tested investigated
Planche et al. (7, 8, 31)					
Meridian Premier		Cytotoxicity test with or without toxigenic culture	95%	97%	
TechLab Quick Check		Cytotoxicity test with or without toxigenic culture	84%	100%	
Remel Xpect		Cytotoxicity test with or without toxigenic culture	82%	96%	
Meridian Immunocard		Cytotoxicity test with or without toxigenic culture	90%	99%	Systematic review using data from different publications. Use of different reference systems (cytotoxicity test or toxigenic culture) ignored.
BioMérieux VIDAS		Cytotoxicity test with or without toxigenic culture	76%	93%	
Nowak-Weekley (27)					
Meridian Bioscience, Inc., Cincinnati, OH	432 samples	Toxigenic culture	58%	95%	CCFA plus enrichment broth used as reference
Alcala et al.(26)					
X/pect	367 samples	Cytotoxicity test with or without toxigenic culture	49%	96%	CLO Agar from Biomerieux 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 (32)					
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 toxigenicity testing by Ridascreen
Ridascreen, r- Biopharm	256 patients		**51%**	98%	
CDSA-Agar	256 patients		70%	100%	Improved 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