Infection Control & Hospital Epidemiology (2020), **41**, 1148–1153
doi:10.1017/ice.2020.267

Original Article

Sequential introduction of a multistep testing algorithm and nucleic acid amplification testing leading to an increase in *Clostridioides difficile* detection and a trend toward increased strain diversity

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Abstract

Background: Most clinical microbiology laboratories have replaced toxin immunoassay (EIA) alone with multistep testing (MST) protocols or nucleic acid amplification testing (NAAT) alone for the detection of *C. difficile*.

Objective: Study the effect of changing testing strategies on *C. difficile* detection and strain diversity.

Design: Retrospective study.

Setting: A Veterans' Affairs hospital.

Methods: Initially, toxin EIA testing was replaced by an MST approach utilizing a glutamate dehydrogenase (GDH) and toxin EIA followed by *tcdB* NAAT for discordant results. After 18 months, MST was replaced by a NAAT-only strategy. Available patient stool specimens were cultured for *C. difficile*. Restriction endonuclease analysis (REA) strain typing and quantitative in vitro toxin testing were performed on recovered isolates.

Results: Before MST (toxin EIA), 79 of 708 specimens (11%) were positive, and after MST (MST-A), 121 of 517 specimens (23%) were positive ($P < .0001$). Prior to NAAT-only testing (MST-B), 80 of the 490 specimens (16%) were positive by MST, and after NAAT-only testing was implemented, 67 of the 368 specimens (18%) were positive ($P =$ nonsignificant). After replacing toxin EIA testing, REA strain group diversity increased (8, 13, 13, and 10 REA groups in the toxin EIA, MST-A, MST-B, and NAAT-only periods, respectively) and in vitro toxin concentration decreased. The average $\log_{10}$ toxin concentration of the isolates were 2.08, 1.88, 1.20 and 1.55 ng/mL for the same periods, respectively.

Conclusions: MST and NAAT had similar detection rates for *C. difficile*. Compared to toxin testing alone, they detected increased diversity of *C. difficile* strains, many of which were low toxin producing.

(Received 2 April 2020; accepted 20 May 2020; electronically published 24 June 2020)

Since the late 1980s, enzyme immunoassay (EIA) for toxins A and B have been the primary method for laboratory detection of *C. difficile*.[1] EIA offered laboratories a relatively rapid test compared to older cell cytotoxicity assays.[2] However, toxin A and B EIAs were plagued by variable detection sensitivity; reports indicate that the sensitivity varies from 47% to 83%.[3,4] These variable sensitivities in testing make it difficult for hospitals to determine the number of true *C. difficile* infections (CDIs).[5]

To overcome the relative insensitivity of toxin EIAs, many clinical microbiology laboratories adopted a multistep algorithm. A glutamate dehydrogenase (GDH) assay was added as the initial screening test in combination with toxin A and B EIAs. GDH has a much higher sensitivity as a common antigen found in all *C. difficile* isolates (toxigenic and non-toxigenic), but it lacks the specificity of the toxin EIA.[2,4] In combination, GDH testing and toxin A and B EIA resulted in increased sensitivity and specificity for *C. difficile* diagnosis.[2,4,5] In 2009, *C. difficile* detection via nucleic acid amplification testing (NAAT) became commercially available.[2] The most commonly used gene target for testing is *tcdB*, which corresponds with the potential for toxin B production.[2,6,7] In a study comparing 9 toxin detection assays, *tcdB* NAAT had similar specificity compared to cell cytotoxicity assay and cytotoxigenic culture.[4] However, because of the increased sensitivity with NAAT, it has a limited capacity to differentiate between CDI and asymptomatic carriage of *C. difficile*.[8]

As *C. difficile* testing strategies continue to evolve, data regarding the impact these different testing strategies have on *C. difficile* strain diversity detection are limited. In this study, we investigated

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Cite this article: Skinner AM, *et al.* (2020). Sequential introduction of a multistep testing algorithm and nucleic acid amplification testing leading to an increase in *Clostridioides difficile* detection and a trend toward increased strain diversity. *Infection Control & Hospital Epidemiology*, 41: 1148–1153, https://doi.org/10.1017/ice.2020.267

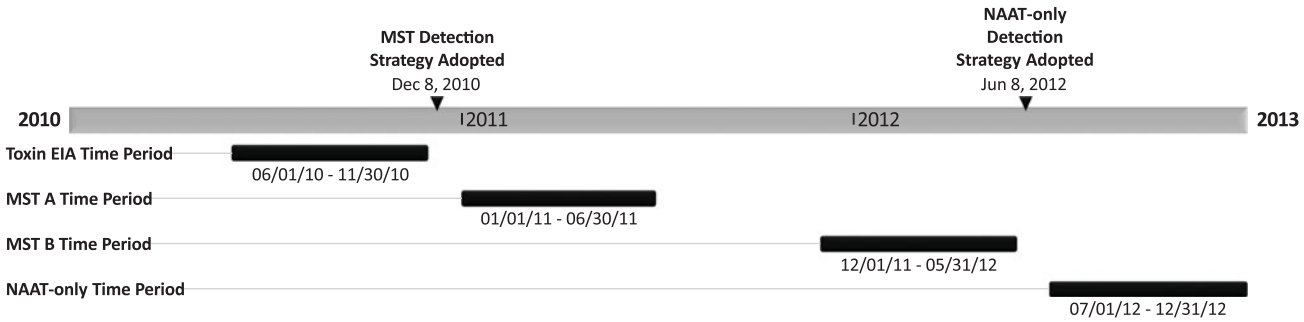

Fig. 1. Graphical representation of the periods analyzed and when the *C. difficile* testing strategies were implemented. Note. EIA, enzyme immunoassay; MST, multistep testing; NAAT, nucleic-acid amplification test.

the impact that testing strategy changes had on *C. difficile* detection as well as the strain diversity of the *C. difficile* isolates detected.

Methods

In 2010 and 2012, the Edward Hines, Jr, Veterans' Affairs Hospital underwent sequential changes to *C. difficile* testing protocols. Prior to December 2010, *C. difficile* was detected via toxin A and B EIAs (Vidas, bioMèrieux, Marcy-l'Étoile, France). In December 2010 a multistep approach (MST) was adopted for the detection of *C. difficile*. GDH and toxin A/B EIA (*C. diff* Quick Chek Complete, Techlab/Wampole, Blacksburg, VA) were used as initial screening tests. Specimens that were positive for *C. difficile* by GDH but negative by toxin EIA were further analyzed by molecular testing for *tcdB* by polymerase chain reaction (PCR, GeneXpert, Cepheid, Sunnyvale CA). Notably, in December 2010, testing was further restricted by no longer allowing >1 stool specimen per patient per week to be tested for *C. difficile*. Another change in testing strategy took place in June 2012, with the introduction of NAAT-only testing (GeneXpert, Cepheid) to replace the MST protocol.

We conducted a retrospective analysis of *C. difficile* positive stool specimens comparing the 6 months prior to and the 6 months after testing protocols were changed (Fig. 1). The 6-month periods were labeled by testing modality: (1) the toxin EIA period (June 1, 2010, through November 30, 2010), (2) the MST-A period (January 1, 2011, through June 30, 2011), (3) the MST-B period (December 1, 2011, through May 31, 2012), and (4) the NAAT-only period (July 1, 2012, through December 31, 2012). The 1-month washout periods between both testing changes, December 2010 and June 2012, were excluded from analysis.

All stool samples that tested positive for *C. difficile* in the clinical laboratory were frozen for subsequent testing. In the 6-month period in which toxin EIA was the only method of detection, there were 79 positive *C. difficile* tests and 57 specimens were available with sufficient amount of stool for further analysis. In the 6-month study period after the implementation of MST, there were 121 positive *C. difficile* tests. Of these 121 positive tests, 91 specimens had adequate stool for further analysis. In the 6 months prior to changing from the MST strategy to a NAAT-only testing strategy, there were 80 positive *C. difficile* tests, of which 49 specimens had sufficient stool for further analysis. During the 6 months after the NAAT-only testing strategy was adopted, there were 67 positive *C. difficile* tests with 39 specimens available for further testing. Patients with recurrent episodes of *C. difficile* within the study period were excluded from further analysis.

Culture

Frozen stool samples from patients that tested positive for *C. difficile* were thawed and inoculated on taurocholate-cefoxitin-cycloserine-fructose agar plates (TCCFA) and were incubated for 48–72 hours in an anaerobic chamber. After incubation, distinct colonies with typical *C. difficile* morphology were subcultured onto BBL anaerobic blood agar and were incubated anaerobically for 48–72 hours. After duplicate stool specimens were excluded, *C. difficile* was recovered from 39 of 46 stools prior to the MST testing algorithm, from 67 of 70 stools after the addition of the multistep testing, from 49 of 80 stools prior to NAAT-only testing, and from 39 of 67 of stools after changing to the NAAT-only testing strategy. The *C. difficile* isolates were frozen at −80°C prior to subsequent analysis.

Restriction endonuclease analysis typing

Restriction endonuclease analysis (REA) typing was performed on the recovered *C. difficile* isolates by *Hind*III digestion of total cellular DNA.[9] DNA fragments were separated by electrophoresis on a 0.7% agarose gel, as previously described.[9] The resulting *Hind*III restriction patterns were compared with previously characterized strains. Patterns showing a 90% similarity index were placed in the same REA group (letter designations). Identical patterns were given specific REA type (subgroup) number designations (eg, BI 12, BI 11, etc). The most common BI group pattern includes 3 very closely related patterns and is designated as BI 6/8/17.

Quantitative in vitro toxin production

One isolate from each non-BI group and 1 isolate from each BI subgroup were selected during each 6-month period for quantitative toxin production. The *C. difficile* isolates were incubated on BBL anaerobic blood agar for 48 hours. Colonies of *C. difficile* were then placed into brain heart infusion (BHI) broth and incubated anaerobically for 48 hours. The samples were then centrifuged, and the supernatants were separated. The supernatants were tested for total toxin concentrations using a toxin A/B kit (Tech Lab, Blacksburg, VA) as recommended by the manufacturer. Concentrations were extrapolated from a standard curve created using purified toxin A of known concentrations. Low toxin levels were arbitrarily defined as $<\log_{10}$ 2.5 ng/mL (316 ng/mL), and very low toxin levels were arbitrarily defined as $<\log_{10}$ 1.1 ng/mL (12.6 ng/mL).

https://doi.org/10.1017/ice.2020.267 Published online by Cambridge University Press

Table 1. Number of Tests Performed and Results During Sequential Toxin EIA, Multistep Testing (MST), and Nucleic Acid Amplification (NAAT) Testing Periods

Variable	June 1, 2010 to November 30, 2010		January 1, 2011 to June 30, 2011			December 1, 2011 to May 31, 2012		July 1, 2012 to December 31, 2012		
	Toxin EIA Period No. (%)	No. Per 30 Days	MST-A Period No. (%)	No. Per 30 Days	*P* Value	MST-B Period No. (%)	No. Per 30 Days	NAAT-only Period No. (%)	No. Per 30 Days	*P* Value
Total tests performed	708	116.7	517	86.2		490	80.8	368	60.3	
EIA negative	629 (88.8)	103.7	372 (72)	62.0		380 (77.6)	17.1			
EIA positive	79 (11.2)	13.0	68 (13.2)	11.3		34 (6.9)	5.6			
Positive GDH/ negative toxin EIA			77 (14.9)	12.8		76 (15.5)	12.5			
NAAT negative			24 (31.2)	4.0		30 (39.4)	4.94	301 (81.8)	49.3	
NAAT positive			53 (68.8)	8.8		46 (60.5)	7.6	67 (18.2)	11.0	
Total positive tests	79 (11.2)	13.0	121 (23.4)	20.2	.0001[a]	80 (16.3)	13.2	67 (18.2)	11.0	.52[b]

[a]The rate of positive stool tests increased significantly between Toxin EIA and MST-A periods.
[b]The rate of positive stool tests did not increased significantly between MST-B and NAAT-only periods.

Results

Change from toxin EIA to MST diagnostic testing

From June 1, 2010, to November 30, 2010 (toxin EIA period), 708 stool specimens were tested and 79 specimens tested positive for *C. difficile* by toxin EIA (11%; 95% confidence interval [CI], 9%–14%) (Table 1). During this time, the average number of positive *C. difficile* tests was 13 and the average total number of tests was 116.7 per month (Table 1). From January 1, 2011, to June 30, 2011 (MST-A period), 121 of 517 total stool specimens tested were positive for *C. difficile* (23%; 95% CI, 20%–27%; $P < .0001$ for the toxin EIA period vs MST-A period comparison). During the MST-A period, toxin EIA was positive for 68 specimens of 121 positive tests. (56.2%; 95% CI, 47%–65%). The remaining 53 positive tests were determined by NAAT to resolve discrepant GDH–EIA results. Positive *C. difficile* tests were more frequent during the MST-A period, with 20.2 per month, but the total number of tests performed also decreased (Table 1).

In total, 105 *C. difficile* isolates were strain typed by REA; 39 isolates prior to MST (toxin EIA period) and 65 isolates after MST(MST-A period). During the toxin EIA period, a total of 8 different REA group strains were identified (Fig. 2). During the MST-A period, 13 different REA groups were detected. In addition, an organism other than *C. difficile* was identified in 2 of the 66 stool specimens that tested positive by the MST protocol.

Overall, 18 isolates (46.2%) from the toxin EIA period and 36 isolates (55.4%) from the MST-A period belonged to the epidemic REA BI group. The BI 6/8/17 subgroup was the most frequently identified BI subgroup before and after the change in testing, consistent with previous epidemiological studies at our institution.[10]

Representative *C. difficile* isolates from each REA group before and after the implementation of MST were analyzed for quantitative toxin production in vitro (Fig. 3). The average $\log_{10}$ toxin concentration during the toxin EIA period and MST-A period were 2.08 ng/mL and 1.88 ng/mL, respectively (Fig. 3). The largest range of toxin production occurred during the MST-A period (0–3.69 ng/mL) (Fig. 3). The BI group strains had the highest toxin levels throughout the study (Fig. 4). Prior to the MST protocol, 5 of 8 REA strain groups had low toxin production ($<\log_{10}$ 2.5 ng/mL), and after the change to an MST algorithm, 9 of 13 REA strain groups demonstrated low toxin levels. Of these low toxin producers, 1 REA strain group was deemed a very low toxin producer ($<\log_{10}$ 1.1 ng/mL) during the toxin EIA period, and 2 REA strain groups were deemed as such during the MST-A period.

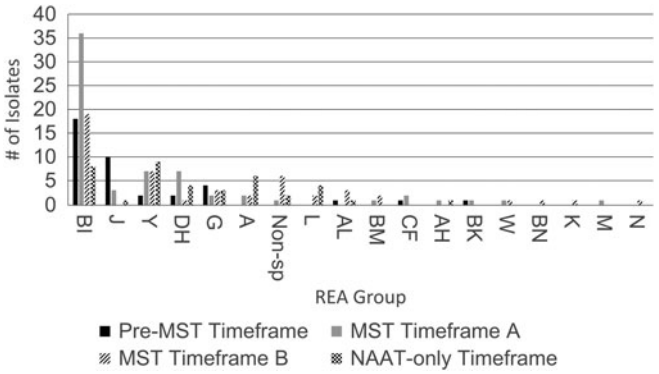

Fig. 2. Frequency and distribution of *C. difficile* REA group strains during 4 separate periods. Toxin EIA period represents the 6 months prior changing to a multistep testing (MST) diagnostic algorithm using EIA toxin as the sole diagnostic method. MST-A period represent the 6 months after changing to an MST algorithm. MST-B period represents and the final 6 months of using the MST algorithm. NAAT-only period represents the 6 months after the test CDI testing strategy changed to nucleic acid amplification testing only.

Change from MST to NAAT-only diagnostic testing

From December 1, 2011, to May 31, 2012 (MST-B period) 490 stool specimens were tested by MST, yielding 80 positive *C. difficile* tests (16%; 95% CI, 13%–20%). Toxin EIA was positive in 34 tests, and the remaining 46 were detected by NAAT. From July 1, 2012, to December 31, 2012 (NAAT-only period) 368 stool specimens were tested, yielding 67 positive tests (18%; 95% CI, 15%–23%; $P = .52$). Compared to the MST-B period, the total number of tests per month during the NAAT-only period decreased from 80.3 to 60 tests.

In total, 88 *C. difficile* isolates underwent REA typing during these 2 periods: 49 isolates from the MST-B period and 39 isolates from the NAAT-only period. Moreover, 13 different REA groups were detected during the MST-B period and 10 REA groups were detected in the NAAT-only period (Fig. 2).

https://doi.org/10.1017/ice.2020.267 Published online by Cambridge University Press

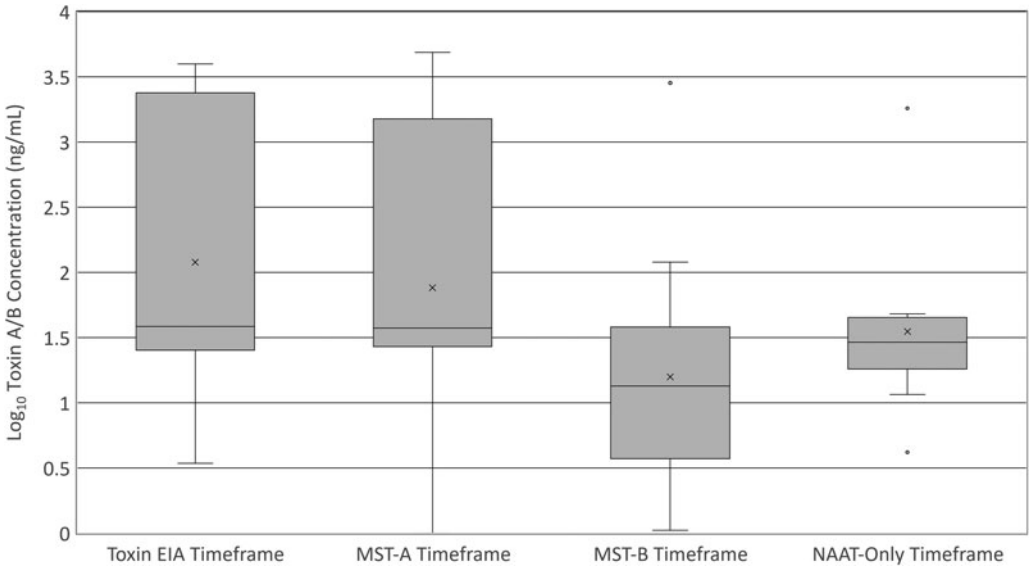

Fig. 3. A box-and-whisker plot of the log_{10} total toxin A/B concentration (ng/mL) of each representative REA group strain from each period. The X indicates the mean during each period. Outliers represented by °. During MST-A period, 2 isolates had no toxin production by in vitro analysis which were identified as a nontoxigenic REA group M and a non-specific REA pattern strain.

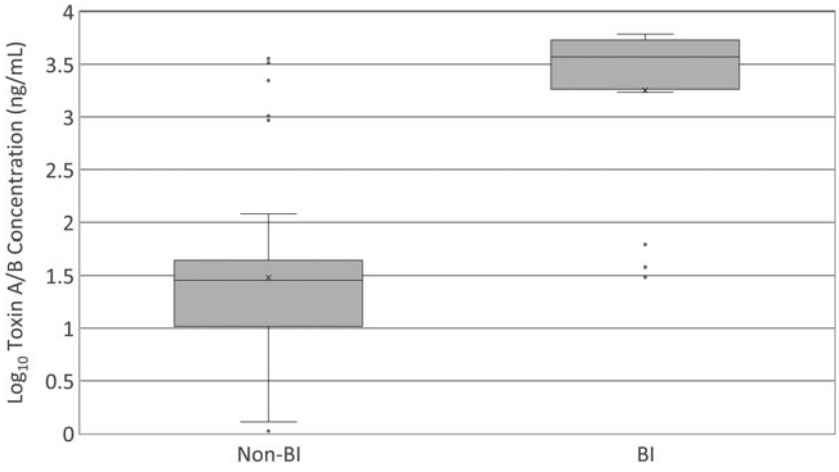

Fig. 4. A box-and-whisker plot of the *in vitro* Log_{10} total toxin A/B concentration (ng/mL) of REA group BI strains and Non-BI group strains. The X indicates the mean during each period. Outliers represented by °.

Of the isolates that underwent REA typing, 19 isolates (38.8%) were typed as REA group BI during the MST-B period and 8 isolates (20.5%) were classified as REA group BI during the NAAT-only period. This reflects a 47.2% decrease in REA BI group isolates from MST-B period to the NAAT-only period ($P = .10$). REA BI 6/8/17 accounted for 16 of 19 and 4 of 8 BI isolates during the MST-B period and NAAT-only period, respectively.

Representative *C. difficile* isolates from each REA group before and after the implementation of NAAT-only test strategy were analyzed for quantitative toxin production *in vitro* (Fig. 3). During the MST-B period and the NAAT-only period, REA BI isolates were the only strain group not to be labeled as a low-toxin group. The average log_{10} toxin concentrations were 1.20 ng/mL and 1.55 ng/mL during the MST-B period and the NAAT-only period. In the final 6 months of the MST testing protocol, 12 of 13 REA group strains were classified as low toxin producers, and after the change to a NAAT-only testing strategy, 9 of 10 REA group strains were classified as low toxin producers. These findings are reflected in a narrower distribution of in vitro toxin quantification (Fig. 3). Of these low toxin-producing strains, 6 were very low toxin producers during the MST-B period and 2 strain were labeled as such during the NAAT-only period.

Twelve-month epidemiologic comparison

When comparing the first 12 months (toxin EIA period and MST-A period) to the second 12 months (MST-B period and NAAT-only period), REA group BI *C. difficile* isolates decreased from 51.9% to 30.7% ($P = .0034$) and REA group J decreased from 12.5% to 1.1% ($P = .002$). Conversely, REA group Y and nonspecific REA groups increased from 8.7% to 18.2% ($P = .056$) and from 0.96% to 9.1% ($P = .0124$), respectively.

Discussion

From 2010 to 2012, our hospital underwent sequential methodologic changes for detecting *C. difficile*, similar to the changes made in clinical laboratories across the United States during this period.[11,12] After incorporating diagnostic testing strategies with increased sensitivity, we noted an increased detection rate of *C. difficile* that was associated with a trend toward increased strain diversity. These changes were most notable after switching to the MST algorithm where NAAT was used to resolve discrepancies between discordant GDH and toxin EIA results. After changing from an MST algorithm to a NAAT-only testing protocol, the detection rate and strain diversity did not change appreciably.

https://doi.org/10.1017/ice.2020.267 Published online by Cambridge University Press

These data revealing increased detection rates are consistent with previous findings that MST algorithms and NAAT-only testing are much more sensitive in the detection of *C. difficile*.[2–5] The implementation of both the MST and the NAAT testing protocols at our hospital led to an increase in the rate at which *C. difficile* was detected despite a decrease in the number of stools tested, further validating the increased sensitivity of these tests. Our findings suggest that the higher detection rate was driven by the detection of additional *C. difficile* strains not recovered by toxin testing alone.

After the implementation of high-sensitivity testing strategies, strain diversity nearly doubled and remained elevated throughout the remainder of our study. The trend toward increased strain diversity corresponded with an increase in the detection of isolates that we characterized as low and very low toxin producing. After the implementation of the MST algorithm and the eventual transition to a NAAT-only protocol, low-toxin strains accounted for the majority of isolates recovered.

Changes in the epidemiology of CDI may have influenced the findings our study. The epidemic REA group BI was the most common strain at our hospital 3 to 4 years prior to this study accounting for 72% of first CDI episodes.[10] We noted a progressive decrease in the percentage of BI during the transition from MST testing to NAAT testing in this study. After MST had been in place for nearly a year, the number of stools tested were nearly identical (85.7 and 80.3 per month for MST-A and -B periods, respectively), but the number of positive *C. difficile* decreased by 30.3% ($P = .005$). We postulate that this decrease in detection is linked to the decrease in REA group BI isolates, which decreased by 40.8% between the two 12-month intervals. REA group BI, which corresponds to PCR ribotype 027 and NAP1 by PFGE, has notable high levels of toxins A and B in vitro and was responsible for multiple hospital outbreaks of severe disease in the early 2000s.[13,14]

Following the decrease of REA group BI in the latter half of our study, most isolates recovered were low and very low toxin-producing strains (Fig. 3). We postulate that detection of low toxin-producing strains accounted for the increased sensitivity of testing during the MST periods when nearly 66% of the positive test results required NAAT to settle a discordant GDH-positive/toxin EIA-negative test result.

The second most common group strain in this study was REA group Y, which is historically among the more common strains identified in North America.[15] Our data show that toxin production in this strain group was variable, ranging from high toxin to very low toxin production in vitro. Although they are not typically associated with significant morbidity and mortality, REA group Y is a fully toxigenic strain carrying both *tcdA* and *tcdB* genes and is associated with clinical disease.[13,16–18]

The MST algorithm and the NAAT-only testing strategy were both more sensitive than toxin EIA testing, but they do have potential drawbacks. As with any test, as sensitivity increases, the chance for a false-positive test increases as well.[19] The presence of the *tcdB* gene does not always correlate with active *C. difficile* infection and could indicate colonization with *C. difficile* and diarrhea due to another cause.[20–23]

For these reasons, the IDSA/SHEA CDI guidelines recommend using NAAT-only testing when there is an institutional agreement to limit testing to patients not on laxatives who have new onset of significant diarrhea.[24] In addition, limiting testing for outpatients to those with risk factors such as antibiotic and healthcare-associated exposures should improve specificity.

Recently, increasing evidence has supported toxin EIA testing in conjunction with NAAT testing.[25] A recent study of close to 5,000 patients found that stools that test positive by GDH and toxin EIA reflexed to NAAT for discrepant results were associated with increased risk for recurrence as well as greater severity of disease compared to NAAT alone.[25] Since this study by Guh et al, some hospitals have implemented the use of *C. difficile* toxin testing to confirm a positive *C. difficile* NAAT test.

The limitations of our study include potential confounding by the changing epidemiology of *C. difficile* over time as noted above for strain REA group BI. Other limitations include potential confounding by changes in clinical testing practice, such as the limitation on the number of stool tests submitted per week between the toxin EIA and MST-A periods. The number of positive tests decreased significantly between the MST-A and MST-B periods, suggesting a possible population bias. We tried to minimize this bias by focusing our analysis on the immediate periods before and after changes in diagnostic tests.

In conclusion, our results confirm that toxin EIA testing alone lacks optimal sensitivity to detect *C. difficile* when compared to high-sensitivity testing strategies such as MST and NAAT-only protocols. More notably, our results reveal a possible increase in *C. difficile* strain diversity when changing from a toxin EIA strategy to an MST algorithm. In contrast, the change from MST to NAAT-only testing was not associated with any significant change in detection rate for *C. difficile* or change in strain diversity suggesting that MST algorithms that use NAAT for resolution of discrepant GDH/toxin EIA results are comparable in sensitivity to NAAT-only testing if they are used in the appropriate clinical setting. The increased sensitivity of these test may be related to the detection of low toxin-producing isolates. Further investigation is needed to determine whether detection of these low toxin-producing strains correlate with clinical infection or colonization.

Acknowledgments. We thank Stacey Spadoni and Bruce Dunn from the Hines VA clinical microbiology laboratory and the VISN12 pathology service line for their help and for facilitating this study. We also thank Susan Sambol for help with the in vitro toxin testing and David Lyerly for supplying the toxin A standard for the quantitative toxin assay.

Financial support. Dr Stuart Johnson and Dr Dale Gerding are supported by the US Department of Veterans' Affairs Research Service.

Conflicts of interest. All authors report no conflicts of interest relevant to this article.

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https://doi.org/10.1017/ice.2020.267 Published online by Cambridge University Press