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Review Article

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Diagnosis of Clostridium Difficile: A Wrong Algorithm and an Improvement in Immunoassays

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ABSTRACT
We compared the 2-step and 3-step algorithms for diagnosis of Clostridium difficile infection (CDI) in clinical cases between October and December 2023. We observed in 3-step algorithm 22 false positives (FP) among the 62 positives (35%), compared to 40 positives without FP in 2-step algorithm. 30 All FP are positive for glutamate dehydrogenase (GDH) and Toxin A and/or B immunochromatography (IC), and negative for toxigenic C.difficile PCR. When processing the samples with a solution of leukocidins and Methicillin Resistant Staphylococcus aureus (MRSA) the FP to GDH is negative, as well as FP from different immunoassays. The cause of FP is an interspecies cross-reaction between human leukocytes and mouse immunoglobulins, a method-dependent error (immunoassay) that increases the 35 probabilities of FP in the 3-step algorithm. Modified algorithms and sample processing with MRSA and leukocidins allow equivalent results to the 2-step algorithm, with a reduction in the total number of molecular tests required (47-61%).

Since the appearance of diagnostic algorithms for CDI, these have been modified and adjusted according to scientific evidence and diagnostic needs until reaching those currently used and recognised, the main ones being the 2-step and 3-step algorithms, the latter being considered non-inferior, faster and less costly [1]. When clinical evidence of CDI is found, samples are sent to the laboratory and, if they are adequate, they are analysed to make a diagnosis. In our department, a 3step diagnostic algorithm was used:

- First step: GDH IC (negative is reported, positive at the second step).
- Second step: Toxin A and/or B IC (GDH+ and Toxin+ reported positive; GDH+ and Toxin- at the third step).
- Step 3: Molecular test by polymerase chain reaction (PCR) for toxigenic C.difficile (Genexpert C.difficile/Epi, CEPHEID) with result reported. The 2-step algorithm does not use Toxin IC, it uses GDH IC and if positive PCR. The immunoassays used were biotical C.difficile GDH card and biotical C.difficile Toxins A+B card, both from Biotical Health. In May 2022, in the microbiology laboratory of Hospital San Cecilio, we observed three doubtful.

60 positive cases in a few days. After reviewing the clinical manifestations of the patients and the results of the cultures, we suspected a diagnostic error: Salmonella enterica was isolated and identified in one case and Campylobacter spp. in the other two. Although cases of coinfection are known, three cases in a week is very unusual for a microbiology service that covers 400,000 people, with more than 200 cases investigated per month and a prevalence of 9%. PCR for toxigenic C.difficile was performed on 65 the three doubtful samples and the results were negative.

A retrospective analysis of the last month found three other confirmed cases of misdiagnosis: preserved samples were PCR negative for toxigenic C.difficile. Six other suspected cases were classified as "probable" FP because no specimen was recovered: they had other viral or bacterial infections that 70 could explain the clinical manifestations, or responded well to treatments not suitable for CDI.

These 12 cases had in common that they did not fit the classic epidemiological criteria for CDI: hospitalisation, surgery and/or previous antibiotic treatment. Our department established

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special surveillance for cases with these characteristics, checking the positive cases with PCR.

Between June and September this special surveillance detected 10 FP cases allowing them to be correctly reported and one probable FP, the sample was lost. Upon review of the 10 FP, one of these did not meet the epidemiological criteria: it had recent previous hospitalisation and antibiotic treatment.

At the same time, these clinical samples were frozen to find out where the error was, after ruling out IC batch problems. In October, the commercial company repeated CI and PCR by a different method (Allplex GI Bacterial I Assay, SEEGENE) on 10 of these samples. The PCR results were the same as those obtained previously by Genexpert, negative for CDI, and coincided with the cultures: Allplex GI 85 also detects other pathogens such as Salmonella, Yersinia, Shigella and Campylobacter. Not so the result of the GDH and Toxin IC: only 2 of the 10 samples were still GDH positive, and of these only one was also Toxin positive.

Faced with these errors of the 3-step algorithm, the management of the service allowed a90 changes in the diagnostic method from October, although IC Toxin was not stopped, the diagnosis was a2-step algorithm: GDH, negative is reported, and positive is PCR to decide the diagnosis (regardless of the result of the IC Toxin).

Why were samples previously positive and now not positive? Freezing of samples and duration95 of freezing. The samples that remained positive for GDH were the most recent ones: they had been kept for less time frozen. The interference causing FP was sensitive to freezing. Another clue was provided by a review of the 23 cases detected (16 FP and 7 "probable" FP): 11 cases with positive cultures for enteropathogenic bacteria (6 Campylobacter, 4 Salmonella and 1 Shigella); and among the remaining cases 3 were diagnosed with inflammatory bowel disease (IBD), 2 were cross-reactive for100 other IC (Rotavirus, Adenovirus and Norovirus) and another case in which a parasite study was requested showed abundant leucocytes under the optical microscope (OM).

Most of the samples were inflammatory diarrhoea. What if all these FP were caused by the presence of leukocytes in the stool? Proving this was105 complex because leukocytes are not observable by OM in all samples and their presence in suspected cases of CDI is normal. To test this hypothesis, we needed sample processing to remove leukocytes without altering GDH and Toxin: after processing, repeat IC in true positives (TP) would remain positive and in FP would be negative. Although freezing had guided us, it is not a good experimental model (slow, laborious and unreliable). Heating was not considered because of denaturation and other110 problems related to sample handling. The use of monoclonal antibodies, biological agents, ionising radiation, acids, bases, salts, etc. were ruled out for various reasons.

In a microbiology laboratory there is a simple and safe way to lyse leukocytes: Methicillin Resistant Staphylococcus aureus (MRSA). All S.aureus produce at least 3 types of leukocidins (HlgAB, HlgCB, and LukAB/HG), while the most virulent isolates produce up to 5 types (the above plus PantonValentine leukocidin [PVL], and LukED) [2]. These leukocidins are bicomponent water-soluble proteins excreted by the bacteria, with the ability to recognise leukocytes, insert into their membranes and generate pores, which ultimately lyse them. LukAB/HG is slightly different from the others, existing as a stable heterodimer in solution and establishing a reservoir of toxin on the surface of the bacterium. In 120 addition, most of the immunoassays used have been tested with different bacteria, including S.aureus, and cross-reactivity has been ruled out.

Since October, all GDH IC positive samples were subjected to: Toxin IC, PCR for toxigenic
C.difficile and GDH IC after processing the sample with a suspension of leukocidins and MRSA (see125Methods). With this processed sample, Toxin IC was repeated on an ad hoc basis due to lack of resources.

Results

A total of 660 samples of suspected CDI cases were processed between October and December 2022, a total of 77 samples were GDH positive: 38 Toxin positive and 39 negatives. Only 3 of these 77 samples (3.9%) were grossly purulent and/or bloody.

Of the 38 GDH+/Tox+ samples: 16 were positive by PCR and 22 were negative (among the latter 3 were very purulent and/or bloody).

Of the 39 GDH+/Tox- samples: 24 were PCR positive and 15 negatives.

A total of 40 cases were reported as positive. In contrast, the 3-step algorithm would have resulted in 62 positive cases (38 GDH+/Tox+ plus 24 GDH+/Tox-/PCR+), with an error of 35% (22 FP).

Regarding the GDH performed after sample processing with MRSA and leukocidins (MRSA-GDH):

Of the 38 samples +GDH/+Tox: 100% concordance with PCR.

Of the 39 +GDH/+Tox samples: 92.3% concordance. The 24 PCR-positive samples were also positive for MRSA-GDH; but among the 15 PCR-negative samples there were 3 positives for MRSA-GDH.

Modified algorithms (figure 1 and 2) with this sample processing obtain the same results as the 2step algorithm, and with a reduction in total PCR needed between 44-64% (table 1). We have included as a paradox the diagnosis of CDI in a single step, using only MRSA-GDH determination: only 3 FP compared to the 22 FP of the 3-step diagnosis, and without the need to use a single PCR.

Tab 1:

31/12/22	GOLD STANDAR				PARADOX?
Algorithm	**2-steps**	**3-steps**	**2-steps+MRSA**	**3-steps+MRSA**	**1-steps+MRSA**
Positives	40	62	40	40	40
TP	40	40	40	40	40
% TP	100,00	64,52	100,00	100,00	93,02
FP	0	22	0	0	3
% FP	0,00	35,48	0,00	0,00	6,98
Total PCR	77	39	43	27	0,00
% red. PCR	0	49,35	44,16	64,94	100,00

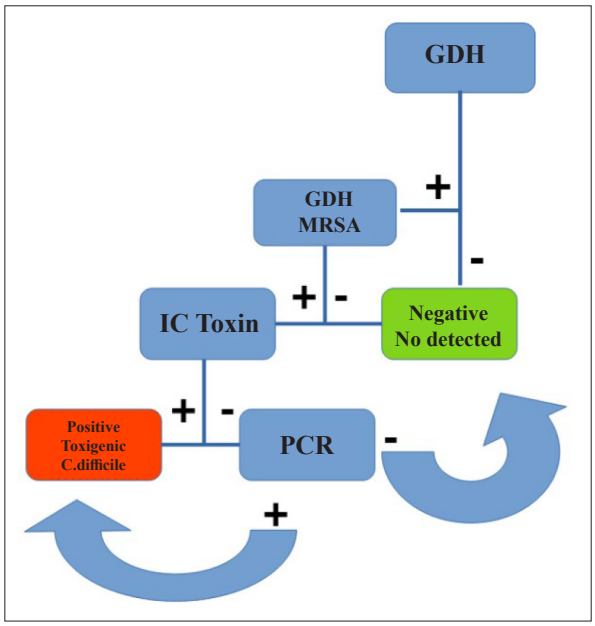

Figure1: Modified 3-steps algorithm.

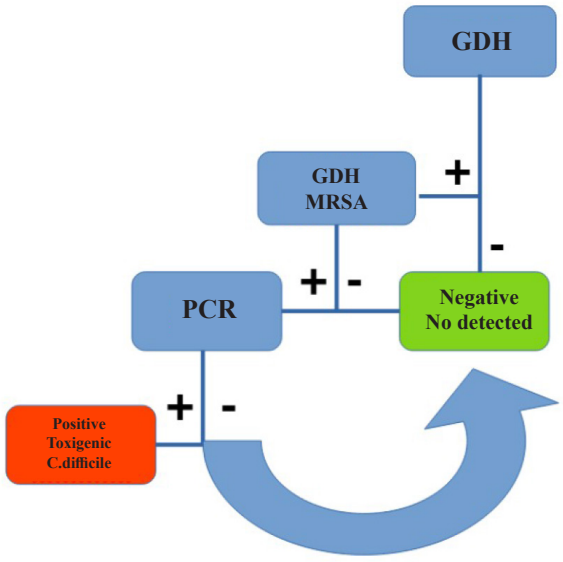

Figure 2: Modified 2-steps algorithm.

With 40 TP among 660 samples, we have a prevalence of 6%, down from 7.5-9% in previous months. Among the 22 GDH+/Tox+ FP, a total of 8 cases were patients with hospitalisation, surgery and/or recent previous antibiotic treatment.

Estimating 100% sensitivity in the GDH IC and the 2-step algorithm as the gold standard,160 although it is not correct, we can calculate theoretical data such as the false positive ratio (FPR) that allow us to discuss the results:

GDH IC specificity: 583/ (583+37) = 0.9403
3-step algorithm specificity: 598/ (598+22) = 0,9614 165 -
MRSA-GDH IC specificity: 617/(617+3) = 0.9951
GDH IC FPR = 0,0597
3-step algorithm FPR = 0,0386

Discussion

Among the studies of ICD diagnostic methods since 2009, we have not been able to find any that compare the 2-step and 3-step algorithms exactly as we know them today. There are some similar175 studies, which are not comparable for different reasons: low sample size or testing by cell cytoxicity neutralization assay (CCNA) on GDH+/Toxin+ results instead of PCR [10-16]. Although years ago, the CCNA was considered the gold standard, it is a subjective method, not very reproducible and gives results after 24-48 hours [15-17]. These studies are used in other subsequent studies in which 2-step algorithms are evaluated to argue for the use of 3-step algorithms, which would allow similar results to be obtained with a lower PCR cost,although without experimentation[11-14]. Thus, we found a meta-analysis with a theoretical construction of a 3-step algorithm on 10,000 samples, in which method errors are considered as independent and with a prevalence of 5%, with specificities of 0.87 for the GDH test and 0.96 for the Toxin test: 432 VP and 49 FP (similar total of FP in our study, but with 15 times less sample size) [9]. We observed that 3-step algorithms, where the first step was PCR did not present this problem: the total error frequency is reduced as the product of the independent error frequencies of each method (PCR vs. IC) [21].

With our results we can think that we use a Toxin IC with very low specificity if we consider as in other studies that the errors of each IC are independent of each other, the total error frequency is 190 given by the multiplication of the individual error frequencies for each method [9]. For the FP, this frequency is the false positive ratio (FPR) that we can calculate with the specificity. As all the FP in the 3-step algorithm are GDH+/Toxin+, we can infer that the FPR of the 3-step algorithm is equal to the product of the FPR of GDH IC by the FPR of Toxin IC, so we can calculate it and infer from it the "theoretical" specificity of the Toxin IC.

FPR Toxin IC x FPR GDH IC = FPR3-step algorithm; FPR Toxin IC = FPR 3-step algorithm/ FPR GDH IC FPR Toxin IC = 0.0386/0.0597 = 0.64 → "Theoretical" specificity Toxin IC = 0.36

Although some Toxin IC may be less specific, this one in particular would beat all known IC in the ranking of worst specificity. We know that this is not the problem: first, because experimentally this IC (biotical C.difficile Toxins A+B card) has never had such low specificity values. And second, the errors of these two methods cannot be considered as independent because experimentally we have shown that they are related, even if different antigens are detected. They are very similar methods and share 205 an intrinsic structural error in their design: interference by cross-reactions of human leukocytes with mouse antibodies, which is also the main cause of FP. For this reason, if this error occurs in a GDH IC for a sample, it is much more likely that the same error occurs in the same sample for the Toxin IC: the Toxin IC of the FP were also negative after processing for leukocidins and MRSA, although due to lack of resources this was only performed on some samples.

Estimating a Toxin IC specificity range of 0.94-0.84 (FPR = 0.06-0.16), if the method errors were considered independent, the expected FPR of the 3-step algorithm would have been between 0.00350.0095; a value 11 to 4 times lower than the real value obtained. If for a sample a method-dependent error occurs in an IC, the probability of the same method-dependent error occurring in a B IC, instead 215 of decreasing, as they are related and dependent errors, increases, and in the case of GDH and Toxin in the order of 11 to 4 times over the expected frequency.

We wonder how it is possible that such an unreliable diagnostic method as the 3-step algorithm (one out of three positives is wrong) has been used for so many years. The answer is complex, and is not only due to considering the GDH and Toxin immunoassay FP as independent and unrelated errors. It is an algorithm with high sensitivity, few false negatives (FN). A large number of samples are processed, with more than 90% being negative, so that any specific findings can be minimised among the total number of samples processed. Moreover, the general consensus on this point has always been diametrically opposed: the suspected error in the use of immunoassays for GDH 225 and Toxin was underdiagnosis, not overdiagnosis [15].

If detecting diagnostic errors in 3 steps is difficult, it is also difficult to associate them with their cause. The use of leukocidin and MRSA in this study makes it possible to see a relationship between the presence of leukocytes in faeces (inflammatory diarrhoea) and FP, but it is impossible without this processing: one of the causes of inflammatory diarrhoea is CDI [3-5]. Not all inflammatory diarrhoeas are FP and not all FP are inflammatory diarrhoeas.

This interspecies cross-reaction between human leukocytes and mouse immunoglobulin (IG) has been reported before, and does not occur between any leukocyte and any mouse IG, it depends on the individual, the leukocyte and the type of IG [6]. We can observe that leukocidin and MRSA processing also negativises the GDH of Tox- samples (12 out of 39 GDH+/Tox-): in these samples, the leukocytes cross-react with the IG of the GDH IC, but not with the IG of the Toxin IC. We could not observe the effect of leucodidin and MRSA processing in all Toxin+ IC due to lack of resources, but in the FP where we did, Toxin negativised. The result of this difficulty in targeting the problem is in the partial solution we took between June and September: a 3-step algorithm checking positive results with PCR based on clinical/ epidemiological data surveillance. Ten FP were detected, although only 9 would have been detected if one of these cases had not been assigned by mistake to the group without hospitalisation, without surgery and without previous 245 antibiotic treatment. The results obtained from October to December reminded us of an old laboratory axiom "where you don't look, you don't find": of the 22 FP, only 14 belonged to this special surveillance group while 8 were cases with hospitalisation, surgery and/or recent prior antibiotic treatment.

When we asked ourselves whether the problem was due to a specific type of immunoassay (GDH IC in use) or to the structural design of all the immunoassays, the punctual tests with the Toxin IC indicated that it was a structural design error. We found that this problem was also present in other immunoassays with samples giving FP: in faeces for Rotavirus, Adenovirus, Astrovirus, Norovirus type I, Norovirus type II and Giardia lamblia ICs; and in respiratory samples for Respiratory Syncytial Virus, Influenza A, Influenza B and SARS-Cov-2 ICs. In all of them, the processing of the sample with leukocidins and MRSA negatived the FP result of the immunoassay, coinciding with the result obtained by molecular biology techniques. Although we could also suspect it for Helicobacter pylori IC in stool when negative with leukocidins and MRSA in some samples, we lacked a confirmatory method.

Since 2010, the potential FP of the 3-step algorithm has been systematically underestimated. The main problem was the sequential use of two immunoassay methods, similar in performance and also in their intrinsic errors. As these errors are the most important, they have been considered as independent errors, when they are not. By considering the total error frequency as the product of the individual error frequencies of each method, the FP ratio (frequency) of the 3-step algorithm was underestimated: instead of decreasing in proportion to the specificity of both methods and having a frequency of 0.005, the errors 265 committed decrease in a much smaller proportion and are 8 times higher than expected.

The use of animals other than mice to obtain IG for immunoassays does not appear to avoid these interspecies cross-reactions, IG from other animals (rabbits, pigs, hamsters, cats, horses) are able to bind human leukocytes [7,8].

Processing samples with leukocidins and MRSA allows for a 92% reduction in GDH FP (from 37 to 3) enabling accurate diagnosis with less use of PCR (44-64% reduction, depending on the algorithm used, fig.1 and 2), and we have indications that its application to other immunoassays will also increase the performance and reliability of these assays. This increase in specificity of immunoassays using 275 leukocidin and MRSA sample processing is an overall improvement for diagnosis, not only qualitatively in terms of accuracy and speed, but also in terms of ease of use and resource savings.

As to how feasible it is to undertake a modified algorithm using leukocidin and MRSA sample processing in a microbiology laboratory in the short to medium term: it is unlikely. Although the use of 280 MRSA and leukocidins is straightforward and the use of liquid cultures is less labour intensive than PCR, this processing is not yet commercially available in a kit or alongside immunoassays.

The main biases we could not avoid are the use of the 2-step algorithm as the gold standard and not testing leukocidin and MRSA processing in all Toxin-positive IC. Lack of resources prevented us 285 from ascertaining the cause of the 3 FP for GDH IC after leukocidin and MRSA processing.

Conclusions

We strongly discourage the use of CDI 3-step diagnostic algorithm. We recommend the use of a 2-step diagnostic algorithm, until a standard leukocidin and MRSA sample processing system is available that allows the use of modified algorithms, which is expected to increase the speed and accuracy of diagnosis at lower cost.

The use of this sample processing in immunoassays other than GDH detection will allow faster, more accurate and lower cost diagnostics than current ones, although further research and validation is needed before they can be used in diagnostics.

Methods

The IC used were biotical C.difficile GDH card and biotical C.difficile Toxins A+B card, both from Biotical Health, used according to the instructions provided[18,19]. The PCR method used was Genexpert C.difficile/Epi (Cepheid), used according to the instructions provided[20].

Processing of Samples with Leukocidins and MRSA

A pathogenic clinical strain of Methicillin Resistant Staphylococcus aureus (MRSA) is used. The culture is isolated on blood agar (BA) or blood chocolate agar (BCA), and subcultured on BA, BCA, BHI medium or any other medium that allows MRSA to produce leukocidins, at 37°C for 48h or more (depending on inoculum and total volume, 48 hours to prepare 10 ml in liquid BHI, with 12 more hours of culture more than one litre can be prepared in liquid BHI). As leukocidins are produced only in the stationary growth phase, their concentration will increase over time. Afterwards, they can be stored at room temperature: solid media for 3-4 weeks, until the drying of the colonies makes their handling difficult, and in liquid media for more than 6 months without losing their properties (tested by the author), although their stability is probably even higher.

There are two Options for The Treatment of Each Sample:

(a). From culture on solid medium: resuspend 5-10 colonies of the culture on blood agar in 0,5 ml of the buffer used for the immunoassay (failing this, PBS), to a McFarland > 4.

(b). From liquid culture: resuspend 0,5 ml of the culture on BHI (bacteria tend to settle to the bottom) in 0,5 ml of the immunoassay buffer (failing PBS).

The volumes and amounts are standard, but should be adjusted depending on the type of sample and immunoassay. In both ways we obtain a buffered solution with MRSA and leukocidins in suspension. The procedure does not differ at this point:

- The sample is added, as specified by the procedure of the immunoassay technique to be used: the amounts are usually between 150 to 50 microlitres, to avoid the dilution effect they can be doubled.
- Mix and vortex intensively for 30 seconds.
- Incubate at 42°C for one hour, or 37°C for one and a half hours. In totally purulent or bloody samples the incubation period should not be less than 3 hours (incubations of more than 6 hours do not 335 negative true positives).

After these steps, the immunoassay protocol for detection or quantification of the test analyte is resumed.

Conflicts of Interest

Author declares no conflicts of interest. The procedure "Processing of samples with leukocidins and MRSA" is pending a patent application in the name of the author Juan Luis Recio-López.

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