Same faeces, same lab, different methods and some different calprotectin results

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Introduction

Faecal calprotectin (FC) has recently become widely used in primary care to differentiate between irritable bowel syndrome (IBS) and inflammatory bowel disease (IBD) following NICE diagnostic guidelines (DG11)[1]. Obviating the need of inconvenient and costly colonoscopies in patients with normal FC values and no red flag symptoms.

Calprotectin is a member of the S100 protein family capable of binding calcium, zinc and manganese. It is the chelation of manganese which produces its antimicrobial properties after release by various inflammatory cells including neutrophils[2].

There are 10 different ELISA assays registered with the UK NEQAS FC scheme and an additional point of care lateral flow device. All these methods require a preliminary faecal extraction step. There are various extraction methods available and that used, is not usually specified by the laboratory. The longest standing and presumed gold standard is the weighing method[3].

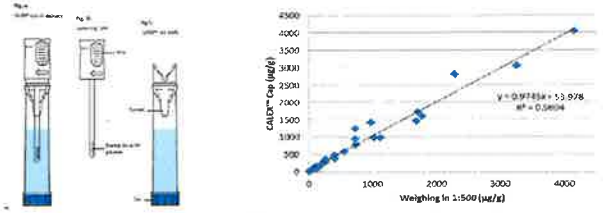
We wanted to evaluate a newly introduced Quanta-Lite FC assay (Werfen Ltd, UK) with Eurospital (ES) extraction tubes and compare it to our routine Bühlmann assay with Bühlmann Calex Cap (BC) extraction tubes to assess the different extraction tubes and ELISA assays on the same faecal sample by the same operator.

Methods

76 routine faecal calprotectin requests from primary and secondary care were used in the evaluation. Samples were received <72hrs from collection and frozen upon receipt. The samples were thawed at 2-8ºC overnight then equilibrated to room temperature for 60 minutes before extraction. The same 76 faecal samples were extracted initially using the BC tubes and then the ES tubes as per the manufacturer's instructions. Once extracted FC was then measured on the Dynex technologies DS2 platform, using the BC tubes with the Bühlmann assay (linear range 10-600µg/g or 30-1800µg/g) and the ES tubes with the Werfen assay (linear range 1 - 400µg/g). The two extraction tubes and methods were evaluated for time and ease of use as well as the FC results obtained by the two different ELISA assays.

Overview Bühlmann Calex extraction tubes:

The FC BC tubes (Figure 1a) contains the correct measured amount of extraction solution for use with the Bühlmann ELISA FC assay. It also has the ability to be used as the primary tube on all of the ELISA analysers available. The plastic shaped rod contains two sets of four grooves which collects 10µg of faecal sample. This BC tube has been found to have a very high correlation to the weighing extraction method[3] which is reported to be the gold standard (figure 1b).



1a 1b

Figure 1:a) An image of the Bühlmann Calex (BC) extraction tube. b) A graph showing the correlation between the BC tube and the 'gold standard' weighing method.

Extraction procedure:

The white sampling pin is removed and dipped into the faecal sample it is then rotated to ensure that all of the 8 grooves are filled completely. This is then re-introduced back into the extraction tube allowing the excess sample to be removed by the presence of a funnel. The tube is then vortexed for 30 seconds and allowed to stand for 10 minutes. If the grooves are not free of faeces after the first vortex, the vortex step is repeated until the faeces is removed from the grooves completely. For samples of a liquid consistency, 10µl of sample is added directly to the extraction tube. Harder samples are pressed against the sample container and the sampling pin is rotated in the sample until all of the grooves are completely filled. The tubes are finally centrifuged for 5 minutes at 500 x g producing a 1:500 dilution of faecal matter ready for FC ELISA analysis. Once extracted the extracted sample is stable at room temperature for up to 3 days, at 2-8ºC for 7 days and at -20ºC for 24 months.

Overview Eurospital Extraction tubes:

The ES tubes contain 2.5ml of extraction solution. The plastic shaped rod houses four radial grooves which aids in the collection of 60µg of faecal sample (figure 2). At the lower end of the extraction tube the end is removed where the sample is then decanted into a suitable tube for analysis. The end also contains a filter which entraps faecal matter from reaching this secondary tube.

Extraction procedure:

The sampling stick is dipped into the faecal sample and rotated to ensure the four radial grooves are completely filled. Any excess sample is removed by rotating the stick on the internal wall of the sample container. The sampling stick is then screwed into the extraction tube and closed. For samples which are liquid in consistency, 60µl of sample is added directly into the extraction tube. For harder samples, 50-100µl of saline is added to the faecal sample and left at room temperature for 60minutes. This is then extracted as above. Each extraction tube is then vortex mixed for 30-60seconds to homogenise the content then placed on a roller shaker for 20minutes. After the roller shaker, ensuring that all the grooves are free of faecal matter, all samples are centrifuged at 3000 rpm for 5 minutes upside down.

The sample extract is then transferred from the ES tubes by breaking the tip at the bottom of the tube and then squeezing the ES tube, squirting the supernatant into a suitable tube for FC ELISA analysis. Care has to be taken not to dislodge the pellet at the other end of the tube. The secondary tubes then require centrifuging again at 3000 rpm for 5 minutes, in case any faecal matter was transferred prior to FC analysis. Once extracted the extracted sample is stable at 2-8ºC for 7 days and at -20ºC for 24 months.

Figure 2: The eurospital extraction device.

Results

Extraction tube comparison:

Advantages of BC relative to ES tubes:

- Only 10µg instead of 60µg of faecal sample required for analysis.
- Easier to fill the grooves in the BC tubes compared to the ES tubes.
- When placing the sample collection stick into the extraction tube excess sample around the stick is easily removed by BC funnel whereas the ES funnel is not snug potentially causing inefficient removal of excess faeces, causing potentially variable faecal extraction.
- Fewer steps in the extraction procedure, the ES tubes require an additional roller mixer step for 20 minutes which holds 12 samples at a time. Meaning for a full ELISA an additional 140 minutes as well as the extracted sample must be transferred into another tube suitable for analysis on an ELISA platform.
- The BC extraction is in a closed system whereas the ES tube is required to be manually squeezed which lead in some instances to splashing of the faecal extract.
- Once extracted BC samples can be stored at room temperature for up to 3 days.

Disadvantage of the BC relative to ES tubes :

- *None.*

Figure 3: A table showing the 76 FC results and how they are interpreted by the two assays. Green - normal, orange - borderline and red – abnormal.[4]

Sample	Bühlmann	Werfen
1	6.66	1.00
2	10.00	1.12
3	11.92	3.95
4	15.33	0.53
5	16.18	5.84
6	18.63	3.21
7	19.29	3.18
8	22.06	6.23
9	22.85	5.73
10	23.05	8.17
11	23.95	7.71
12	26.36	8.37
13	26.70	9.43
14	27.67	9.03
15	28.80	13.63
16	29.43	6.09
17	30.05	8.50
18	30.33	11.95
19	31.45	11.02
20	31.64	21.41
21	33.64	17.52
22	34.01	17.63
23	37.38	10.95
24	41.30	17.77
25	42.92	17.06
26	44.01	14.78
27	45.35	14.34
28	46.02	18.27
29	47.96	24.73
30	48.42	17.89
31	49.56	6.99
32	52.54	24.12
33	55.05	18.38
34	57.09	20.83
35	59.78	45.67
36	60.43	19.43
37	66.92	37.45
38	75.41	32.60
39	76.31	28.66
40	82.80	24.29
41	86.65	25.19
42	89.79	20.57
43	91.36	37.40
44	93.49	17.91
45	98.44	46.17
46	106.55	36.06
47	136.03	
48	142.29	
49	149.72	
50	174.37	43.43
51	183.73	
52	203.89	
53	214.94	
54	219.78	
55	220.61	
56	256.66	
57	303.33	
58	319.80	161.23
59	321.79	
60	350.07	
61	464.19	
62	469.13	
63	522.79	
64	531.48	182.65
65	>600	169.05
66	>600	174.79
67	>600	204.70
68	>600	254.08
69	>600	297.15
70	>600	291.69
71	>600	296.31
72	>600	320.49
73	>600	373.54
74	>600	>400
75	>600	>400
76	>600	>400

Method comparison:

The equation of correlation for the 76 patient samples was Werfen = 0.27 Bühlmann + 6.5, r^2 = 0.811 (Figure 4). Hence the Bühlmann assay results are on average 3.0 times higher than the Werfen assay. At present within our laboratory results of >49µg/g are categorised as abnormal (A) and a referral to Gastroenterology is suggested. The Bühlmann method states an elevated level of between 50-200µg/g may represent mild disease with further investigations required. The Werfen assay reports results of <50µg/g as normal (N), 50-120µg/g as borderline (B) with re-testing in 4-6 weeks suggested and results >120µg/g as A. The assays categorised the 76 samples as follows: Bühlmann, N 40.8% and A 59.2% and Werfen N 61.8%, B 19.7%, A 18.4%. With the current interpretation 59% of the samples analysed by the Bühlmann method were abnormal whereas only 18% of samples tested with the Werfen kit were deemed abnormal (Figure 5). In order to have better interpretative agreement between the assays a borderline range with the Bühlmann assay should be 159 - 415µg/g and positive > 415µg/g.

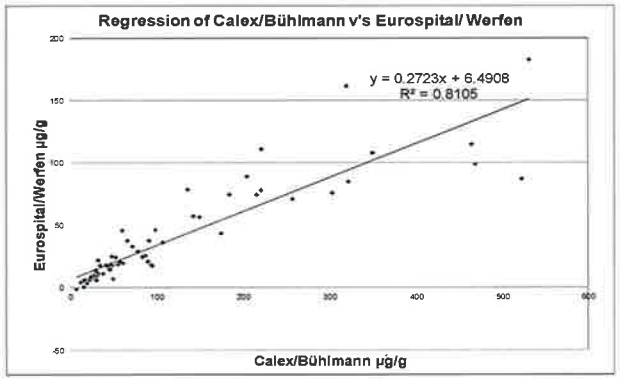


Figure 4: Linear regression of faecal calprotectin measured by the Bühlmann with BC tubes and Werfen with ES tubes.

		Calex / Bühlmann		
		Abnormal	Normal	Total
Eurospital / Werfen	Abnormal	14	0	14
	Borderline	15	0	15
	Normal	16	31	47
	Total	45	31	76

Figure 5: Table of agreement by interpretative categories obtained on the 76 faecal calprotectin samples as measured by the Bühlmann and Werfen assays

Discussion/Conclusion

Overall the ES extraction tubes had more disadvantages than the BC tubes, being more laborious with an unacceptable risk of splashing the extract. Werfen have taken this constructive feedback and decided to try to use a different extraction tube or to try to redesign the extraction tubes. The assays had reasonable over all agreement (r^2 = 0.813) however this did lead to different patient management for 40.8 % (31/76) of the patients due to different results and different interpretative cut-offs. Patients were less likely to be immediately referred to secondary care using the Werfen assay. This may be related to different assay calibration. We recommend that each assay should be audited in relation to clinical outcome and cut-offs changed as indicated. Furthermore the FC ELISA method as well as the extraction method should be reported with the result.

References

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