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Preliminary evaluation of a new *Schistosoma* Immunochromatographic Test

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ABSTRACT

Over 90% of schistosomiasis infections occur in sub-Saharan Africa. A rapid ICT test would be a cheap and easy tool that could be used also in the field. We preliminarily evaluated the performance of a new *Schistosoma* black-latex based IgG-IgM ICT (Black-ICT) on serum samples. The results indicate a high sensitivity (98.0%) but the specificity depends on the application of a cut-off value that can discriminate between positive and negative samples. Considering a possible direct application of this test on blood from finger prick, the results are promising, providied that a signal intensity scale is developed, guiding the result interpretation.

1. Introduction

Schistosomiasis, caused by the digenetic blood flukes *Schistosoma mansoni, S. haematobium* and less frequently by other species, is a neglected tropical disease (NTD) affecting over 200 million people worldwide, with more than 90% of infections occurring in sub-Saharan Africa (Hotez et al., 2019; WHO, 2020).

A free-swimming larva cercaria, released from the intermediate host, a freshwater snail, penetrates the skin of the final host, including humans, and develops to male and female adults, who live in the blood vessels. The eggs, released by the females, are expulsed with feces or urine, depending on the *Schistosoma* species. When the conditions are optimal the released eggs hatch and the miracidia larvae penetrate the snail tissue, where they complete the cycle (WHO, 2020). Schistosomiasis is prevalentin rural communities, especially farmers, anglers and washerwomen, nevertheless it is also reported in urban realities, and inadequate hygiene habits are indeed risk factors of infection (WHO, 2020). During the last decade, Europe has faced a huge wave of migration from low-middle income countries (LMIC), and the environmental changes could possibly promote the spread of infectious diseases worldwide. Temperate and tropical strains of *Schistosoma* can survive the cold Mediterranean winter and permanently establish autochthonous *foci.* In Corsica, a hybrid lineage, resulting from the interbreeding between *S. haematobium* and *S. bovis,* was established in 2013 and was possibly transmitted since then through the *Bulinus truncatus*/mammals cycle (Mulero et al., 2019).

Intestinal and urogenital chronic schistosomiasis are quite dissimilar diseases and present different symptoms; in both cases the severe complications, including bladder cancer, hepatic portal hypertension and infertility, are caused by the body reaction to the worms eggs. Most of *Schistosoma* infections are clinically silent for a long time and hence not routinely screened in at'risk populations, and thus an adequate test is required, especially for the latent infection (Beltrame et al., 2017; Buonfrate et al., 2018).

A recent systematic review, considering 88 studies worldwide about strongyloidiasis and schistosomiasis prevalence among migrants born in endemic countries, reported a pooled schistosomiasis seroprevalence of 18.4%, where Sub-Saharan African migrants had the highest seroprevalence (24.1%) (Asundi et al., 2019). Unfortunately, most estimates of schistosomiasis burden derive from observational studies and primarily include refugees from selected countries. Moreover, the substantial differences in prevalence reported by different authors may be due at least in part to the test used to detect the infection.

A sensitive, point-of-care tool (POCT) would be desirable both in endemic countries (e.g. for population mapping, newborn monitoring after Mass Drug Administration or intermediate host control programmes) and for screening of immigrants and refugees in non-endemic countries. The direct observation of eggs in stool or urine is still considered the gold standard for the diagnosis of schistosomiasis but, due to its relatively low sensitivity, this technique underestimates the prevalence. This is particularly true in those areas where the intensity of the infection is middle-low, thanks to the preventive chemotherapy

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strategy (Bergquist et al., 2017; Silva-Moraes et al., 2019). During the last decades, few molecules from the four life-cycle stages (cercaria and miracidia larvae, adults and/or eggs) have been suggested as good candidates for the immunochemical diagnosis of schistosomiasis. Among them, it is worth to remind the adult microsomal antigens, HAMA, MAMA and JAMA respectively for *S. haematobium, S. mansoni* and *S. japonicum* (Al-Sherbiny et al., 1999; Hancock and Tsang, 1986; Tsang et al., 1984), the adult worm antigen (AWA) or the adult worm extract (AWE) (Grenfell et al., 2012), from *S. mansoni* (SmAWE) or *S. haematobium* (ShAWE), with good performances in Western blot and in indirect haemagglutination assay (Bevilacqua et al., 2012; Hinz et al., 2017), the soluble eggs antigens (SEAs) from *S. mansoni*, with a possible use for the differentiation between chronic and acute phase (Hussein et al., 2004), the recombinant protein SmRP26 from *S. mansoni* (Makarova et al., 2005), the recombinant thioredoxinperoxidase-1 from *S. japonicum* (rSjRTPx1) (Angeles et al., 2011) and the recently suggested extracellular vesicles (EVs) proteins from *S. japonicum* (Chen et al., 2020). Some of these molecules are currently used as antigen or part of the antigenic fraction adopted in commercial kits by different manufacturers (Hinz et al., 2017).

The currently available commercial kits (IVD-CE marked) present a wide range of performance. In 2017, with a retrospective study, Beltrame and co-workers compared the performance of four different commercial kits. Among them, the immunochromatographic test (ICT) "Schistosoma ICT IgG-IgM" (LDBIO Diagnostics), based on pink-latex matrix coated with purified antigen from a crude lysate of S. mansoni adult worms (SmAWE) called from here onward "Pink-ICT", showed the best combination of sensitivity and specificity (96% and 83%, respectively) (Beltrame et al., 2017). Those results suggest the possible use of the ICT as a single test for screening subjects at risk. A subsequent study, aimed at verifying the possible application of the Pink-ICT directly on finger prick blood, gave disappointing results, with a dramatic loss of sensitivity (Buonfrate et al., 2018), possibly due to the interference of the hemoglobin color background with the pink-colored latex matrix. Other possible reasons could be attributable to the smaller amount of serum available from a blood drop, meaning a lower amount of immunogenic proteins, (Buonfrate et al., 2018), in addition to a more complex biological matrix in terms of cells and protein quantity, that can interfere with the signal detection. Nevertheless, the so-called Pink-ICT, was intended for use on serum samples and a possible use of it with whole blood was not indicated. The availability of a rapid ICT test, that could be directly used on blood from finger prick, would represent an easy and cheap screening tool.

For these reasons, with the aim to eliminate all kind of possible interferences generated by hemolized sera or whole blood, LDBIO Diagnostics developed a new ICT, not yet commercially available, based on a black colored latex matrix (Black-ICT), intended for use with either serum or whole blood. The new ICT, requires a double volume of whole blood (in comparison with the volume of serum required by the previous test), allowing a balanced number of antibodies to be tested.

2. Material & Methods

This study was conducted according to the STARD 2015 guidelines (Cohen et al., 2016).

2.1. Setting and participants

We evaluated the performances of the Black-ICT testing 100 banked serum samples, available from the previously published study conducted in 2014-2016 by Beltrame and co-workers, where the performance of different diagnostic tests for Schistosomiasis had been evaluated (Beltrame et al., 2017).

Fifty positive and 50 negative samples were selected using the composite reference standard (CRS) defined by the previous study (Beltrame et al., 2017). Briefly, according to the CRS, the positivity was determined by direct detection of *Schistosoma sp.* eggs in stools (*Schistosoma mansoni* or *Schistosoma japonicum*) or in urine (*Schistosoma haematobium*) and/or at least 2 concordant positive out of 4 immunological tests (Urine CCA dipstick test, Bordier ELISA, Schisto II WesternBlot IgG and Schistosoma ICT IgG-IgM (Beltrame et al., 2017). Among the 50 positive subjects (according to the CRS), six had *S. mansoni* eggs, nine had *S. haematobium* eggs and two had both species. Most of the subjects of our cohort were from Africa, one subject was from Bangladesh, one from Brazil, two subjects from Italy and one from Poland. Twelve subjects from our cohort, as is shown in Table 1, presented at least one co-infection with a parasite. In this study, we kept the original ID number published by Beltrame and co-workers in order to retrieve the data for each tested serum. The complete data set is available in the supplementary material of Beltrame et al. (https://doi.org/10.1371/journal.pntd.0005593.s001).

Eight subjects presented two co-infections, while the other 4 subjects presented three to four co-infections. Specifically the co-infections were with *Strongyloides stercoralis* (5 subjects), hookworm (7 subjects), *Mansonella perstans* (2 subjects), *Trichostrongylus* (1 subjects), *Ascaris lumbricoides* (1 subject), *Dicrocoelium dendriticum* (1 subject) and intestinal protozoa such as *Giardia intestinalis, Entamoeba* spp., *Blastocystis, Iodamoeba buetschlii,* (12 subjects). For convenience, we considered the intestinal protozoa as one co-infection.

This study received ethical clearance from the competent ethics committee (Comitato Etico per la Sperimentazione Clinica delle Province di Verona e Rovigo) on 15 May 2019 (study protocol number 27129).

2.2. ICT test

LDBIO Diagnostic, producer of the Black-ICT test, declares that this is coated with purified antigen from a crude lysate of *S. mansoni* adult worms, just as the commercially available, IVD CE marked, Pink-ICT version. The Black-ICT test, kindly donated by LDBIO Diagnostics, was performed according to the manufacturer's instruction. Briefly, 15 μl of serum were dispensed in the sample well followed by 3 drops of the respective eluent and the results were read after a 30-minutes incubation period as described by the manufacturer. Two independent blinded operators interpreted the test results in parallel for each serum. In order to evaluate the signal intensity of the Black-ICT, we developed a reference colorimetric signal scale, using undiluted and 6 diluted aliquots dilutions of a well-characterized positive control serum sample. The dilutions were tested first by Pink-ICT and the optimal dilution scale was applied to Black-ICT. We scanned the cassettes, aligned the windows of positivity of each cassette and printed the colorimetric scale in normal office paper. Thus, we provided it to the two blinded operators. Positive results were scored from 1 to 7 (1 = faint signal and 7 = maximum intensity) being 0 the negative result (Fig 1).

The scale was performed using undiluted and 6 diluted aliquots of positive control serum sample. Positive results were scored from 1 to 7 (1 = faint signal and 7 = maximum intensity) being 0 the negative result.

A test was considered valid if the control band signal appeared. If a colored signal line appeared, the test was considered positive, and we asked the two operators to score the results according to the reference intensity scale. If only the control band appeared then the test was recorded as negative. Tests recorded as equivocal were repeated and, in case of confirmed uncertain result, the data was recorded as equivocal.

2.3. Statistical analysis

The data obtained in the present study were compared with data reported by the previous study published in 2017 (Beltrame et al., 2017). Sensitivity/specificity of the Black-ICT was calculated as the proportion of positive/negative results over all the infected/non-infected samples respectively, according to the CRS

Table 1
ICT results. The CRS from Beltrame et al. 2017 was used as reference values in the subsequent analyses. Pink and Black ICT results were reported as positive/negative (0 and 1 respectively) and Black-ICT scoring for each sample was also indicated, according to the performed colorimetric guide.

			Pink-ICT results (positive/negative)	Black-ICT results (positive/negative)		(signal scoring)	
id	*** Co-infections**	**Schisto CRS**	**P_ Belt17**	**B_ Op1**	**B_ Op2**	**B_Op1_ COLOUR**	**B_Op2_ COLOUR**
1		0	0	1	0	1	0
2		0	0	1	0	1	0
3		0	0	1	1	3	2
5		0	0	1	0	1	0
6		0	0	0	0	0	0
7		0	0	1	0	1	0
8	Ad, ip	0	0	1	0	1	0
10		0	0	1	0	1	0
11		0	0	1	0	2	0
13		0	1	1	1	4	4
16		0	1	1	0	1	0
21		0	0	E	0	E	0
24		0	0	E	0	E	0
28		0	0	1	0	1	0
30		0	0	1	0	1	0
32		0	0	1	0	1	0
39		0	0	1	0	1	0
48		0	0	0	0	0	0
63		0	0	0	0	0	0
64	Al, ip	0	0	1	0	1	0
73		0	0	1	0	1	0
74		0	1	1	1	2	1
79		0	0	0	0	0	0
80		0	0	1	0	1	0
85	T, Dd, ip	0	0	1	0	1	0
100		0	1	1	1	4	4
101		0	0	1	0	1	0
105		0	0	0	0	0	0
106		0	0	1	0	1	0
108		0	0	1	0	1	0
112		0	0	1	0	1	0
115		0	1	1	0	1	0
121		0	0	E	0	E	0
122	Ss, ip	0	1	0	0	0	0
125	Ad, ip	0	1	1	1	2	1
126		0	0	1	0	1	0
128		0	0	1	0	1	0
138		0	0	1	0	1	0
141		0	0	1	0	1	0
147		0	0	1	0	1	0
148		0	0	1	0	1	0
152		0	0	1	0	1	0
160		0	0	1	0	1	0
168		0	0	1	0	1	0
175		0	0	1	0	1	0
180		0	0	E	0	E	0
186		0	0	0	0	0	0
188		0	0	1	1	2	1
189	Ss, ip	0	0	1	0	1	0
190		0	0	1	E	2	E
12	Ss, Ad, ip	1	1	1	1	3	2
15		1	1	1	1	2	3
19		1	1	1	1	4	5
42		1	1	1	1	5	4
62		1 [m]	1	1	1	2	2
65		1	1	1	1	2	3
69		1	1	1	1	2	1
77		1 [m]	1	1	1	4	4
81		1 [h]	1	1	1	3	3
87		1	1	1	1	5	5
102		1 [h]	1	1	1	4	4
107		1	1	1	1	2	1
117		1 [m]	1	1	1	3	4
132		1 [m]	1	1	1	5	7
139		1	1	1	1	5	5
151	Ad, ip	1 [h]	1	1	1	4	4
159		1 [m,h]	1	1	1	3	2
167		1	1	1	1	5	6
172	Ss, ip	1	1	1	1	4	4
184		1 [h]	1	1	1	5	6
216		1	1	1	1	3	2

(*continued on next page*)

Table 1 (*continued*)

255		1	1	1	1	2	3
278		1	NA	1	1	2	1
282		1	1	1	1	3	4
291		1	1	1	1	2	1
295		1	1	1	0	1	0
296	Ad, ip	1 [h]	1	1	1	3	3
297	Ss, Ad, Mp, ip	1 [h]	1	1	1	5	5
303		1	1	1	1	5	6
313		1	1	1	1	2	1
324		1 [h]	1	1	1	2	2
335		1 [h]	1	1	1	4	5
353		1	1	1	1	4	5
368		1 [h]	1	1	1	3	3
378		1	1	1	1	4	4
381		1	1	1	1	3	4
382		1 [m,h]	1	1	1	4	4
385		1 [m]	1	1	1	4	4
387		1	1	1	1	4	5
392		1	1	1	1	2	2
393		1	NA	1	1	2	1
400		1	1	1	1	6	6
413		1	1	1	1	6	7
415		1	1	1	1	4	5
417		1	1	1	1	6	7
432		1	1	1	1	3	3
470		1	1	1	1	2	1
475		1 [m]	1	1	1	4	5
484	Mp, Ad, ip	1	1	1	1	2	1
497		1	1	1	1	4	4

Id: ID from Beltrame et al., 2017; **Schisto CRS:** data from Beltrame et al., 2017 **(**0: negative to *Schistosoma* infection 1: positive to *Schistosoma* infection); letters in superscript indicate the presence of eggs in stool or urine (m= *S. mansoni*, h= *S. haematobium*). **P_Belt17**: Pink-ICT results from Beltrame et al., 2017; **B_Op1**: Operator 1 - Black-ICT; **B_Op2**: Operator 2 - Black-ICT; **B_Op1_COLOUR**: Operator 1 - Black-ICT with signal intensity score; **B_Op2_COLOUR**: Operator 1 - Black-ICT with signal intensity score; **0:** Negative; **1:** Positive; **NA:** Not Available; **E:** Equivocal; **1-7:** Positive band intensity based on chromatic scale. *Co-infection with other parasitosis: **Ss**: *Strongyloides stercoralis,* **Ad***: Ancylostomatidae*, **Mp**: *Mansonella perstans,* ***T****: Trichostrongylus*, **Al**: *Ascaris lumbricoides*, **Dd**: *Dicrocoelium dendriticum,* **ip**: intestinal protozoa.

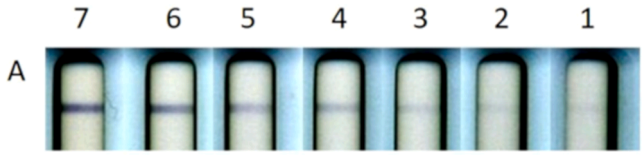

Fig 1. Reference colorimetric signal scale.

indicated by Beltrame and co-workers and uncertainty was evaluated using the 95% confidence intervals. Positive and negative predictive values (PPV, NPV) were estimated based on the CRS values. Agreement between the two operators was evaluated by the Cohen's kappa measure (with 95% confidence interval). The equivocal results to at least one operator were considered as missing values in the statistical analysis. The analyses were performed with the STATA/SE 14.0 software.

3. Results

Two blinded operators interpreted the test. The positive and negative readings, as well as the signal intensity scoring, for the separated operators are reported in table 1.

Five results of the Black-ICT were equivocal/doubtful according to at least one operator. As reported in table 1 all of them were negative at the reference standard. The specificity of the test was calculated including equivocal results in the denominator.

Considering the colorimetric scale, we observed that several false positives, that were scored as faint bands (score = 1) or equivocal by operator 1, were scored as negative by operator 2. Sensitivity and specificity for each reader was calculated using the CRS as reference (table 2).

The agreement between the two operators was also assessed, indicating a low concordance (Kappa index = 0.17). Both analyses were subsequently repeated applying a cut-off threshold at bands scored as faint (score = 1) for operator 1, resulting in an huge increase in the specificity (76%) and agreement (K index = 0.88).

Table 2
Diagnostic performance of the new ICT test.

		Se %	Sp %	PPV	NPV
Pink-ICT*	Beltrame et al. 2017	96 (91-99)	83 (77-87)	78%	97%
Black-ICT	Op_1	100 (92.9-100)	14 (5.8-26.7)	53.8%	100%
	Op_1**	98 (89.4–99.9)	76 (61.8-86.9)	80.3%	97.4%
	Op_2	98 (89.4-99.9)	86 (73.3-94.2)	87.5 %	97.7%

Se = sensitivity; Sp = specificity; () confidence interval; PPV = positive predictive value; NPV = negative predictive value. * "Schistosoma ICT IgG-IgM" (LDBIO Diagnostics), (Beltrame et al., 2017). Op_1: Operator 1; ** : Values obtained applying a cut-off. Op_2: Operator 2.

Among the analyzed samples, 12 patients presented co-infections with other parasites (Table 1): six of them were positive for schistosomiasis, according to the CRS, and six were negative. One sample presenting co-infections with hookworm and intestinal protozoa gave a false positive result according to both operators. No other possible cross reaction was observed.

4. Discussion

The new test confirmed the 50 CRS positive sera, except for one false negative result by one operator. On CRS negative samples we observed a wide difference between the two operators. Contrarily to a very good sensitivity according to both operators (100% and 98% respectively, table 2), the specificity showed a big difference. While it was very low (14%, table 2) for operator 1, for operator 2 it was similar to the Pink-

ICT results (86%, table 2). In contrast to the Pink-ICT, we observed that the Black-ICT presented a weak background signal in several samples, which could mislead the operator.

In fact, the different specificity between the two operators was due to the high number of false positives recorded by operator_1. Analysing the intensity scoring, based on the control colorimetric signal scale we performed, we observed that the majority of these false positive results was registered as a weak band (score =1). This was because operator_1 strictly followed the manufacturer's instructions and recorded as positive also the weak signals. On the contrary, operator_2, based on his own experience, decided to consider the weak bands as background, setting an arbitrary signal cut-off, corresponding to score=1 of the control colorimetric scale (Fig 1). Thus, considering the raw data, the agreement between the two operators was very low (63% Kappa= 0.17). However, applying the same cut off used by operator_2 to operator_1 data, we observed an increase in the agreement between the two operators to 94.00% (Kappa = 0.88). This way the specificity reached a mean value of 81% (Table 2), which is perfectly in line with the previous Pink-ICT data (Beltrame et al., 2017; Buonfrate et al., 2018). Indeed our analysis indicated that a colorimetric reference scale and a signal threshold cut-off is necessary to guide the reading. Some examples are already reported in literature: for instance a 10-point semi-quantitative scale suggested for the Schistosoma POC-CCA (Casacuberta-Partal et al., 2019) or a Smartphone-based reader which will avoid the bias due by the human eye and interpretation (Ong and Poljak, 2019). Among the twelve serum samples from patients with co-infections, only one sample presenting co-infections with hookworm and intestinal protozoa gave a false positive result to ICT tests to both operators. The same sample was also positive at the Pink-ICT in the previous study by Beltrame and co-workers (Beltrame et al., 2017). Considering also that other 5 samples with no co-infection gave false positive results (Table 1), we conclude that, according to the available data, cross-reactivity does not seem to be a major problem.

5. Conclusion

In conclusion, we suggest that the manufacturer include in the kit a control sample to exclude the background signals or even better a colorimetric guide to read and interpret the results especially in those cases where a low intensity infection is more common and the correct interpretation is essential.

Due to the rapidity of execution, the Black-ICT could have the potential to be used as a single test for schistosomiasis in endemic countries with the aim of initial mapping, or else for areas thought to have interrupted transmission, as well as in immigrant and refugees shelters of non-endemic countries. A further prospective study should be indeed planned (with the modifications we are suggesting) for the performance evaluation of the Black-ICT on whole blood from finger prick.

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CRediT authorship contribution statement

Silvia Stefania Longoni: Conceptualization, Data curation, Formal analysis, Investigation, Writing - original draft, Writing - review & editing. **Chiara Piubelli:** Conceptualization, Data curation, Writing - original draft, Writing - review & editing. **Francesca Perandin:** Writing - review & editing. **Eleonora Rizzi:** Investigation, Writing - review & editing. **Nadia Luchetta:** Investigation, Writing - review & editing. **Monica Degani:** Investigation, Writing - review & editing. **Stefano Tais:** Investigation, Writing - review & editing. **Antonio Mori:** Writing - review & editing. **Zeno Bisoffi:** Conceptualization, Supervision, Validation, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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