Calculation of the ELISA's cut-off based on the change-point analysis method for detection of *Trypanosoma cruzi* infection in Bolivian dogs in the absence of controls

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In ELISAs, sera of individuals infected by Trypanosoma cruzi *show absorbance values above a cut-off value. The cut-off is generally computed by means of formulas that need absorbance readings of negative (and sometimes positive) controls, which are included in the titer plates amongst the unknown samples. When no controls are available, other techniques should be employed such as change-point analysis. The method was applied to Bolivian dog sera processed by ELISA to diagnose* T. cruzi *infection. In each titer plate, the change-point analysis estimated a step point which correctly discriminated among known positive and known negative sera, unlike some of the six usual cut-off formulas tested. To analyse the ELISAs results, the change-point method was as good as the usual cut-off formula of the form "mean + 3 standard deviation of negative controls". Change-point analysis is therefore an efficient alternative method to analyse ELISA absorbance values when no controls are available.*

Key words: ELISA - cut-off - change-point analysis - *Trypanosoma cruzi* - dog - Bolivia

In continuous diagnostic clinical tests, the establishment of a reliable cut-off is of paramount importance to discriminate between infected and non-infected individuals. Several standard methods have been proposed to choose optimal cut-offs (Lopez-Raton et al. 2014), and all require known positive and negative individuals to compute the cut-off value that will best discriminate. Enzyme-linked immunosorbent assay (ELISA) is a diagnostic tool carried out commonly in parasitological studies to detect antibodies or antigens related to a specific parasite. They produce absorbance readings, and to discriminate amongst positive and negative results, a cut-off value is needed. The determination of an optimal cut-off value in ELISA assays has long been a concern (Ridge & Vizard 1993). Generally, and especially with home-made ELISAs, cut-off values are estimated using known independent negative sera (sometimes along with positive ones) which are included in the titer-plates amongst the unknown samples. A general formula for a cut-off value is of the form:

$$Cutoff = a.\overline{X} + f\,.\,SD \quad (1)$$

Where $\overline{X}$ is the mean and *SD* the standard deviation of independent negative control readings, and *a* and *f* two multipliers.

Depending on authors, the multipliers can be set arbitrarily, for example to $f = 0$ with $a = 2$ or $a = 3$ (i.e., *cut-off* = twice or three times the mean absorbance obtained from the negative controls), or $a = 1$ with $f = 3$ (i.e., *cut-off* = mean + 3 times the standard deviation) (Classen et al. 1987). However, Frey et al. (1998) claimed that the cut-off can be statistically determined by setting $a = 1$ and $f = t.\sqrt{1 + (1/j)}$ where j is the number of negative controls used in the plate and t is the $(1\text{-}\alpha)^{th}$ percentile of the one-tailed Student t-distribution with $(j\text{-}1)$ degrees of freedom.

To detect infection by *Trypanosoma cruzi*, the causative agent of Chagas disease, Pan et al. (1992) have proposed another formula that takes into account negative and positive controls:

$$Cutoff = X_{neg} + 0.13\,\overline{X_{pos}} \quad (2)$$

Where $\overline{X_{neg}}$ is the mean of the negative controls, and $\overline{X_{pos}}$ the mean of the positive controls.

When no controls are available, the above formulas cannot be used. Change-point analysis is a statistical analysis that can detect in a series of (ascending) values, a step indicating a change. Such change exists in a series of negative and positive ELISA values from a titer plate and should be detected with such an analysis.

The scope of the present study is to evaluate the change-point analysis as a tool to identify positive ELISA reactions when no controls are available. A set of dog sera from a field survey is used to diagnose *T. cruzi* infection and results are compared to those obtained using a standard approach using cut-off values from the usual equations (1) and (2).

doi: 10.1590/0074-02760160119
Financial support: UNICEF/UNDP/World Bank/WHO Special Program for Research and Training in Tropical Diseases/International Development Research Center (TDR/IDRC), "Eco-bio-social research on Chagas and dengue in Latin America and the Caribbean" (Project #A90281), Agence Nationale de la Recherche (ANR) (Project "Alternatives" #ANR-CES2010-CESA018 01).
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Received 21 March 2016
Accepted 10 June 2016

MATERIALS AND METHODS

Negative dog sera - Negative sera were from 16 dogs living in the city of La Paz, where no Chagas transmission exists. Dogs were born in the city and never went out in an endemic Chagas region. Negativity was checked by the Chagas STAT-PAK rapid test which is an accurate test for Chagas diagnosis in dogs (Nieto et al. 2009), and by polymerase chain reaction (PCR) targeting the kDNA of *T. cruzi* following Fernandes et al. (2001), slightly modified by one of us (Aliaga et al. 2011). The 16 negative sera were included as negative controls in each of the processed titer-plates.

Positive dog sera - 10 positive dog sera were obtained from dogs originated from the same region of the field sample (see below) and diagnosed positive both by PCR using the same protocol as above, and by the Chagas STAT-PAK rapid test following the manufacturer's instructions. Then, in each ELISA plate, five-10 of them were included as positive controls to allow the computation of a cut-off value with formula F_3 (Ta s te e I).

Sera of fie eld sample A field sample of 231 dog sera was obtained from four Bolivian populations. Vil-

analysis (for *T. cruzi* identification5() a)-18.7(n)0.9(d 5 m)-30(L i)-22.1(n E)-1.6(D)5.4(T)39.5(A)]TJETEMC /Span <</MCID 235

TMB (3, 3', 5, 5' - Tetramethylbenzidine, SIGMA) was added in each well and the plate was incubated for 5 min at room temperature. Then, 50 µL/well of sulfuric acid 1 N were added to stop the reaction and absorbance values were obtained at 450 nm in a microwell plate reader (Multiskan). The mean absorbance of each pair of duplicate sera was calculated. When the difference between both values was more than 30%, the sample was retested (Lauricella et al. 1998). In total, the 231 dog sera and the controls were processed in seven titer plates.

Cut-off formulas (Table I) - For each of the seven titer-plates analysed, cut-off values were computed using six usual formulas (F_i, i = 1 to 6). The value of the f coefficient in formulas *F4* and *F5* was 2.197 and 3.848 respectively, according to Frey et al. (1998).

Change-point analysis - The whole set of sera was also analysed by change-point analysis which does not need the presence of known positive or negative sera (blind analysis). Change-point analysis is aimed at identifying points in a series where the statistical properties change. In particular, such analysis can be used to detect abrupt steps in the mean level of a series. In the case of ELISA, if absorbance values of a micro-titer plate are ordered in ascending order, negative samples are supposed to be the lower ones in the series while positive ones (if they exist) would be the higher. However, values are not supposed to increase regularly if positive samples exist in the series. Indeed, as positive controls are supposed to be "different" from negative ones, a step, even small, should appear in the series, separating the negative from the positive values. Therefore, change-point algorithms might be used to detect such shihe l-6..7(i)(. Tas)-2.9(t)-22.1(n t)-18.3(hv)16(e v)2.5(a)3.8(s)]TJETEMC /Span <</MCID 432 >>B

TABLE II

Absorbance cut-off values for each of the seven titer plates, computed with formulas F_1 - F_6 and step points detected by the change-point analysis

Plate	F_1	F_2	F_3	F_4	F_5	F_6	Change-point analysis	Highest negative control	Lowest positive control
1	0.519	0.779	0.418	0.441	0.578	0.508	0.471	0.471	0.862
2	0.494	0.741	0.404	0.369	0.461	0.414	0.710	0.380	0.710
3	0.614	0.920	0.473	0.455	0.566	0.509	0.462	0.462	0.941
4	0.503	0.755	0.414	0.374	0.466	0.419	0.496	0.370	0.808
5	0.424	0.636	0.358	0.359	0.469	0.412	0.598	0.343	0.598
6	0.456	0.684	0.384	0.354	0.448	0.400	0.570	0.356	0.843
7	0.552	0.828	0.475	0.415	0.519	0.466	0.432	0.420	1.115

slightly too low, giving some false positive results. Cut-off values from formula F_6 (i.e. with $f = 3$) lie between those estimated with F_4 and F_5. Indeed, because 16 negative controls are used and depending on the confidence level, f is almost < 3 in F_4 and almost > 3 in F_5. With six independent negative controls, f would be 2.777 at the 97.5% confidence level and 6.366 at the 99.9% confidence level. The present results indicate that a f multiplier of at least 3 (as in F_6) should be recommended.

The detection of true positive or true negative individuals is always difficult when individual absorbance values are close to the cut-off value. For that reason, as far as Chagas disease is concerned, the precise detection of cases is usually carried out using several independent assays. For example, ELISA and indirect immunofluorescence assay (IFA) are first carried out, and if the results are not in agreement, a third assay is carried out [r-ELISA (recombinant ELISA) for example]. Although the change-point analysis may detect small changes in a series and has correctly discriminated between the known positive and negative samples of the study, it does not solve the sensitivity problem. False negative or false positive results can therefore exist (as small proportions however). As in a standard procedure, it can be recommended to re-test the samples with other independent assays, in particular those for which the absorbance value lies close to the detected change-point value.

ACKNOWLEDGEMENTS

To the people of Palmarito, Eje pampa, La Brecha and Lagar Pampa, for letting us blood-sampling their dogs, the laboratory personnel of INLASA who helped in field collection of dog blood samples: Guillermo Aliaga, Jaime Carranza and Libia Torrez, and Sam Featherston, from Tubingen University, for correcting the English spelling.

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