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Major factors associated to persistence of bovine trichomoniasis in a mandatory control plan: A eight year retrospective study in La Pampa, Argentine

Jorge Oyhenart

INCITAP - CONICET – Facultad de Ciencias Exactas y Naturales, Universidad Nacional de La Pampa, Av. Uruguay 151, 6300 Santa Rosa, La Pampa, Argentina

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ABSTRACT

Bovine trichomoniasis is a venereal disease caused by the flagellate protozoan *Tritrichomonas foetus*. Infection is related to low conception rates and would have significant impact on calf crop. The state of La Pampa started in 2006 an unprecedented mandatory control program for eradication of bovine trichomoniasis. The compulsory participation of all cattle producers and the yearly control of every bull should be followed by culling of every positive animal. This retrospective study on data from eight years of the control plan showed that 80% of farms had a single year of positive tests. In these farms, positive tests showed a strong decay of disease during the first years that reached a baseline by 2012. A non negligeable proportion of positive bulls in this group can be attributed to false positive tests. Oppositely, farms with two or more years of positive diagnosis accounted for a great proportion of recent cases. These farms were more likely related to less intensive control measures. The non exclusion of carrier bulls is the major factor contributing to the persistance of bovine trichomoniasis.

1. Introduction

Bovine trichomoniasis is a venereal disease caused by the flagellate protozoan *Tritrichomonas foetus*. Low conception rates and long intercalving periods with occassional abortion are distinctive features in *T. foetus* infection (Mylrea, 1962; Clark et al., 1983; Clark et al., 1986). *T. foetus* colonization of the cow genital tract rarely lasts > 120 days (Rae and Crews, 2006). The presence of *T. foetus* is typically asymptomatic in the bull. Young bulls can clear the infection in a short period while aged bulls can become asymptomatic carriers (Clark et al., 1974; Parsonson et al., 1974).

Bovine trichomoniasis has worldwide distribution and is common in regions whith extensive cattle production systems and natural breeding. There is no vaccine to effectively control *T. foetus* host colonization (Gracia Martinez et al., 2018). The treatment of infected animals with nitroimidazoles is effective but it must be performed under controlled conditions. There is general agreement for not using drugs to treat *T. foetus* carrier animals (OIE, 2017).

The most reliable way of controlling trichomoniasis is the use of artificial insemination. In dairy herds from the whole world and meat producing herds from Europe this practice has proven its efficacy (Yule et al., 1989). In other regions it is impracticable. Bovine trichomoniasis control plans undertaken in Australia and the USA included diagnosis and culling of carrier animals. Regulations impose in general that any bull 12 months of age and older that is been imported or changing ownership must have a negative *T. foetus* test within a few days prior to be transported (Ondrak, 2016; OIE, 2017).

Bovine trichomoniasis is endemic in Argentina (Campero et al., 2003). The country adhesion to the International Office for Epizooties rules imposes that every bull for import/export must be free of bovine trichomoniasis. Otherwise bovine trichomoniasis control is mandatory in a single state in Argentina. The government of La Pampa imposed in 2006 a control program for erradication of bovine trichomoniasis and bovine campyloacteriosis (SENASA, 2008). Participation in the program is compulsory for all cattle producers. Every bull in a reproductive herd in La Pampa must be yearly controlled and positive animals must be culled within 120 days. The program reached the full territory by 2008 and by this time > 92% of the herds are yearly controlled.

A recent communication showed that the prevalence of bovine trichomoniasis has decreased in La Pampa since 2008 (Molina et al., 2018). The aim of that study was to predict the time pattern of bovine trichomoniasis and bovine genital campylobacteriosis as well as the association with risk factors over time. However a poor correlation was found between bovine trichomoniasis prevalence and persistence. In this context it was suggested that bovine genital campylobacteriosis and seasonal false negatives tests could be important risk factors for bovine trichomoniasis persistence.

Culling of carrier bulls is the most efficient way of control for bovine

E-mail address: jorgeoyhenart@gmail.com.

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trichomoniasis (Ondrak, 2016). Our thinking is that any attempt to search for other causes of bovine trichomoniasis persistence should study first the degree of adhesion to this "culling rule". Otherwise a screening program must justify the need for a more sensitive detection technique. The balance between benefits, harms and cost-effectiveness largely determine the adherence to the program.

Here, a retrospective study of eight years of positive *T. foetus* tests in bulls and farms in La Pampa was carried out with the aim to identify major factors associated with bovine trichomoniasis persistence. Simple statistic procedures and descriptive analysis were used to demonstrate that non adherence to culling and diagnostic errors were influencing the persistance of bovine trichomoniasis in La Pampa.

2. Methods

2.1. Data

Data were provided by the Veterinary Association of La Pampa and are available under request. Data were collected between 2008 and 2016 and were based on preputial scrapings or washings performed by 385 authorized veterinarians. By 2015 twenty laboratories were authorized to perform the *T. foetus* diagnosis test. The *T. foetus* diagnosis was based on the examination of culture growth for seven days before providing a negative result. The culture was performed in liver infusion medium supplemented with horse serum incubated at 37 °C. There was no information available about proficiency tests.

Data were organized in two datasheets identified as Totals and Positives. The Total Results datasheet was organized as farm producers tested every year with the following column variables: producer ID, Department (administrative division), Year, Field Veterinary Name and ID number for the First and Second Sampling procedure, Number of Animals Sampled in the First and Second Procedure, Farm, Facility Status, First and Second Testing Laboratory, Number of Samples Tested in the First and Second Round and Number of Positive Tests to *T. foetus* and *C. fetus* in the First and Second Round. Data cleaning included the remotion of fourteen rows with missing or duplicated data. The study population consisted of 38,412 entries belonging to 7293 herds tested from 2008 January 1 to 2015 December 31. The producer-ID variable (13-digits code) was used to create the variable "farm" by remotion of the last two digits. The variable "farm" was then summarized in order to bring togheter the animals belonging to every calf producer in a given property. This procedure brought the total of rows for analysis to 33,023. The former datasheets contained animals sampled twice despite having positive results (n = 345) in the first culture test. Former sheets also contained data from farms that were sampled just once (n = 2358). The total of sampled and positive animals were then deduced by taking account of double tests as well as from the exclusion of positive animals.

The Positive Results datasheet consisted of 3395 rows representing every bull with a positive result after *T. foetus* and/or *C. fetus* diagnostic test. Fifteen completely duplicated lines were removed. Bulls with positive tests were defined by: bull ID eartag, producer ID number, Farm name, Department, Year, First and Second Veterinarian Sampler's Name and ID, First and Second Testing Laboratory, and *T. foetus* (and *C. fetus*) test results in the First and Second Test. The positives datasheet was used to build up a categorical variable considering farms with repeated *T. foetus* tests in different years. Then, farms with one or more years with positive animals were categorized according to the number of "repeated-events" (1–5). The Positive Results datasheet was next used to identify farms that did not cull carrier bulls. Eartag numbers including a letter and three numbers for the animal identification were filtered for a minimum of 4 characters. A search for duplicates allowed to identify 15 bulls that were tested twice while being under the same owner, 28 bulls that changed the owner but were still in the same farm and 13 bulls that were sent to a different farm. The farms related to these bulls received the "non_culling" status 1, 2 or 3 respectively.

2.2. Statistical analysis

All statistical analysis was undertaken using R Statistical Software v3.4.4 through the RStudio integrated development environment v 1.1.463 (R Core Team, 2014). Chi-square test, Wilcoxon sign test, Kolmogorov-Smirnov test and Page test were performed with DescTools library or coin package v2.2.1. Linear-by-linear association tests and Cochran–Armitage test were also performed with the coin package. Plots were created with ggplot2 v3.1.0 (Wickham, 2016).

2.3. Ethical statement

The study did not involved the use of human or animal subjects or samples. Data were gently provided by MV D. Dubie from the Colegio de Veterinarios de la Provincia de La Pampa. Procedures are referenced to original works.

3. Results

3.1. Positive bulls and positive farms

Table 1 shows results obtained in 8 years of the bovine trichomoniasis control plan in the state of La Pampa. From 3126 positive bulls affiliated to 1305 farms about one third (1016 bulls, 376 farms) were detected in 2008. Positive cases significantly decreased in 2009 as a consequence of relaxed control measures (Molina et al., 2018). Thereafter, positive cases stabilized in around 315 bulls distributed in 140

Table 1
Results from the compulsory control plan for erradication of *Tritrichomonas foetus* from herds in La Pampa, Argentina. Data from years 2008 to 2015 are shown as tested and positive bulls and farms.

	2008	2009	2010	2011	2012	2013	2014	2015	Total	Mean
Tested farms	3549	2169	3539	4485	4801	4856	4991	4633	33,023	4127.87
Positive farms	373	91	130	151	136	156	129	139	1305	163.12
% positive farms	10.51	4.19	3.67	3.37	2.83	3.21	2.58	3.00	–	4.17
Tested bulls	43,388	23,848	31,681	37,269	39,404	42,206	43,116	41,348	302,260	37,788.00
Positive bulls	1016	220	282	382	280	335	296	315	3126	390.75
% positive bulls	2.34	0.92	0.89	1.02	0.71	0.79	0.69	0.76	–	1.02
Tested bulls positive farms	8828	1941	2391	2704	2224	2873	2517	2997	26,475	3309.37
% total bulls in positive farms	20.35	8.12	7.55	7.26	5.64	6.81	5.84	7.25		8.60
Mean tested bulls per farm	12.22	11.02	8.94	8.30	8.20	8.69	8.63	8.92	–	9.37
Mean tested bulls per positive farm	23.66	21.32	18.39	17.90	16.35	18.41	19.51	21.56	–	19.64
Mean positive bulls per positive farm	2.72	2.41	2.17	2.53	2.06	2.15	2.29	2.27	–	2.33
% positive bulls per positive farm	11.51	11.33	11.79	14.13	12.59	11.66	11.76	10.51	–	11.91

na: non aplicable.

farms by year (Table 1). The proportion of positive tests showed significant variation from 2008 to 2015 (chi-square = 852.4, $p < 2.2e-16$). Although the number of positive bulls and positive farms showed no apparent change during 2010–2015 the number of farms recruited raised and the percent of positive tests decreased (chi-square = 37.8, $p = 4.035e-07$).

The number of bulls tested every year in positive farms experienced a strong reduction to about one third of that registered in 2008 (Table 1). Culling of carrier bulls could account for such a regression. In fact, the fall in the number of bulls in positive farms strongly resembled the fall observed in the number of positive farms and in the number of positive bulls. A two-sample Kolmogorov-Smirnov test supported the idea that both the number of positive bulls and the number of bulls in positive farms were drawn from the same continuous distribution (D = 0.125, $p = 1$). The percent of the total bulls represented in positive farms could be thus directly determining the number of positive events.

The mean number of bulls tested per positive farm per year (mean = 19.64, sd = 2.37) was twice the average of bulls tested per farm every year (mean = 9.37, sd = 1.45) suggesting that false positive tests could represent a significant proportion of the positive records. A Wilcoxon signed rank test for paired values showed significant difference (V = 0, $p = .0078$) suggesting the existence of size effect bias in the diagnostic test.

Thereafter the number of positive bulls per positive farm should show a reduction according to the overall fall in the number of bulls tested. It should also accumulate a difference accounting for culled animals. Nevertheless, the mean number of positive bulls per farm with positive tests was almost static between 2008 and 2015 (Kendall tau: -0.35, 95% CI: −0.88 to 0.17). The percent of positive animals in positive farms did not change either (Kendall tau: -0.07, 95% CI: −0.72 to 0.58).

Fig. 1 depicts the relationships between the percentage of positive bulls, the percentage of positive farms percentage, the percentage of bulls per positive farm and the percentage of positive bulls per positive farm. The values of these variables are shown in Table 1 and were linearly transformed in order to show the same y-intercept in Fig. 1. The Figure illustrates that the percent of positive bulls was tightly associated to the number of tested bulls (see positive bulls, positive farms and bulls per positive farm) suggesting that a great proportion of positives could be false positives. Then Fig. 1 shows that the percentage of positive bulls in positive farms is almoste steady, suggesting that positive bulls were not excluded.

3.2. False discovery rate

Sometimes the time needed to take a second sample from bulls with a negative result in the first diagnosis was shorter than the time allowed to exclude a bull from a herd. Thus data from animals with positive results contained a number of animals with two diagnostic results despite having a positive result in the first diagnosis. Table 2 shows the number of animals with double test results that were positive at the first one, at the second or at both the first and the second tests. These values were used to quantify the possible error in the determination.

From 3126 *T. foetus* carrier bulls detected in eight years 871 were identified in the second test. Otherwise between 2255 first round positive tests 753 samples had a second test evaluation (were not culled or were culled later) and 345 of these were also found positive the second diagnostic test. True positives were then estimated to be 1033 (2255*345/753) and false positives were 1221 (2255*408/753). The false positive discovery rate was then calculated as the ratio between the number of negative results in the first test that were positive in the second test and the total of positive results in second test. The overall false discovery rate estimation for the eight years (2008–2015) was 0,46. The estimated rates for every year from 2008 to 2015 are shown in Table 2. The estimation being based only in the number of double tests can be considered independent of the prevalence of disese. However, as expected, the false positive rate increases as the prevalence of the disease decreases.

3.3. Repeated positive tests

The search for farms having repeated positive results in 8 years showed 291 farms among 1126 *T. foetus* positive. After agreggating farms with positive results, carrier bulls were distributed in 948 farms from which 80,5% had a single year of positive test/s. Repeated years of positive tests found in the remaining 185 farms were distributed as follows: 117 farms had two positive tests, 47 farms had three positive tests, 17 farms had four positive tests and 4 farms had five positive tests in eight years.

Farms with a single year of positive tests and farms with repeated years of positive results were segregated in order to search for their

Fig. 1. Temporal variation in *Tritrichomonas foetus* measures. The relationship between the percentage of positive bulls, the percentage of positive farms, the percentage of bulls per positive farm and the percentage of positive bulls per positive farm is shown. The variables were linearly transformed (values in Table 1 were divided by a factor shown between brackets) in order to show the same y-intercept.

Table 2
False discovery rate in the culture test for *Tritrichomonas foetus* detection in La Pampa, Argentina. The estimated false discovery rate for years 2008 to 2015 is shown below the number of carrier bulls identified and the number of bulls that had two disgnostic tests. Shown are bulls that tested positive in two occasions (1st Positive - 2nd Positive), that had positive test first and had a negative result later (1st Positive - 2nd Negative) or that were positive first and then positive (1st Negative - 2nd Positive).

	2008	2009	2010	2011	2012	2013	2014	2015	2008–2015
Tested bulls	43,388	23,848	31,681	37,269	39,404	42,206	43,116	41,348	302,260
Positive bulls	1016	220	282	382	280	335	296	315	3126
1st Positive - 2nd Positive	20	49	26	33	44	25	29	119	345
1st Negative - 2nd Positive	117	85	94	79	119	70	61	246	871
1st Positive - 2nd Negative	43	49	51	62	45	28	30	100	408
False Discovery Rate	0.37	0.44	0.41	0.48	0.53	0.54	0.45	0.65	0.46

influence in the total positive results (Fig. 2). The number of bulls in farms with a single positive year showed an almost sustained regresion since 2008 (Fig. 2A). A similar picture was obtained with the number of farms in this category (Fig. 2B). Bulls and farms in the single positive year category represented a large fraction of the total positive tests in 2008 but a minor fraction of positives in the last three years. Furthermore the mean number of positive bulls in single positive year farms always represented the lower fraction of positive events (Fig. 2C).

Bulls in farms with repeated positive years represented one-third of the total positive tests in 2008 (Fig. 2A). Farms that would repeat positive years represented one sixth of the total in 2008 (Fig. 2B). Both numbers fell dramatically in 2009 and then went up in 2012, where they represented the highest fraction of positive tests. While the average of farms in this category was 58 only 13 farms were registered in 2010. The mean of positive bulls per farm in this category experienced a sudden burst in 2011 and kept growing in the following years. This population was likely hosting farms that were not in compliance with the culling rule.

3.4. Non-culling effect on Tritrichomonas foetus control

Almost 70% of the carrier bulls detected during 2008 were on farms that had no positive animals in the following years (Fig. 3A). The same category of bulls in farms with a single year of positive tests represented < 30% of the total obtained in the last three years of the control plan (Fig. 3A). Oppositely the bulls from farms with 2–5 positive events became more frequent in the last years. An Asymptotic Linear-by-Linear Association Test supported the hypothesis suggesting a strong association between the two ordered nominal variables ($Z = 16.688$, $p < 2.2e\text{-}16$).

In a further step, a search was conducted to associate repeated positive tests with non culled bulls. Several bulls that tested positive more than once were found among data from the venereal control plan. The search showed 15 bulls that were tested twice while being under the same owner, 28 bulls that changed the owner but were still in the same farm and 13 bulls that were sent to a different farm. The farms related to these bulls were segregated into non-culling categories 1, 2 and 3 respectively. Bulls in farms in these categories were contrasted with the temporal series and with repeated events farms.

Fig. 3B depicts the results obtained after splitting the number of positive bulls detected every year into bulls belonging to farms that were in non-accomplishment with the culling rule. Strickingly bulls in farms from categories 1 and 2 represented approximately 50% of the positive bulls detected in 2010–2011 in good agreement with the burst observed in repeating farms (Fig. 3B,Fig. 2C). Moreover, a close association was found between farms with 1–5 repeated years of positive results and farms in different non-accomplishment categories. A Linear-by-Linear Association Test showed that there was an association between the variables ($Z = 7.1205$, $p = 1.076e\text{-}12$).

4. Discussion

There is no effective vaccine for controlling bovine trichomoniasis and there is no authorized treatment for the infected animal. The efforts to prevent bovine trichomoniasis rely thus on the exclusion of *T. foetus* carrier bulls from a herd (Yao, 2013). The rationale adopted by countries with extensive calf producing systems consists in the restriction of *T. foetus* spread.

The state of La Pampa has undertaken a different approach, that of a compulsory control program that imposes the anual control of every bull in every farm. Bovine trichomoniasis in La Pampa significantly decreased between 2008 and 2011. However there was a less evident regression of both positive bulls and positive farms in the following years. A recent study searched for causes of bovine trichomoniasis persistance and failed to show any relationship between bovine trichomoniasis persistence and bovine trichomoniasis prevalence (Molina et al., 2018).

The low sensitivity of the culture test for bovine trichomoniasis is relatively well known (Cobo et al., 2007; Oyhenart, 2018). Animals with a reduced *T. foetus* load that were not detected in a first test would probably be detected in a second test. In a given farm an animal that did not have a positive test one year would probably be positive later. Thus in the evaluation of diagnostic results obtained systematically for almost a decade, we could suspect that the persistence of the disease is due to the low sensitivity of the analytical technique if positive diagnostics were repeated regularly. However farms with a single episode of positive results represent > 80% of the total farms with bovine trichomoniasis. The low frequency of new positive experiences suggests that the culture technique is sensitive enough to ensure the identification of every carrier bull in most of the farms.

On the contrary, the evidence obtained indicated that a non-negligeable proportion of positive results could be attributed to false positive tests. First, the difference between the mean number of bulls tested per farm and the mean number of bulls tested per positive farm suggests a size effect bias in diagnosis. Second, the percent of positive bulls was associated to the number of tested bulls (Fig. 1). Third, a baseline number of cases indicating disease persistance after 2012 were found in farms without previous or posterior positive tests (Fig. 2). Finally, the lack reproducibility of positive tests suggested that as much as 65% of positive tests could be false positives in 2015 (Table 2).

Flagellate organisms growing in medium for *T. foetus* diagnostic have been well described (Walker et al., 2003). Non specific growth of these and other protozoa could account for as high as 11% positive samples (Sánchez and Boero, 2013). Overgrowth of *T. foetus* like organisms can lead to mistaken positive results. However other errors can lead also to false positive results and they more likely to occurr in large screening programs.

Strictly speaking a screening test is a procedure performed on members of a defined asymptomatic population to assess the likelihood of their members having a particular disease (Maxim et al., 2014). in the screening for *T. foetus* carriers any positive test determines that a given bulls should be excluded. However, by definition screening tests

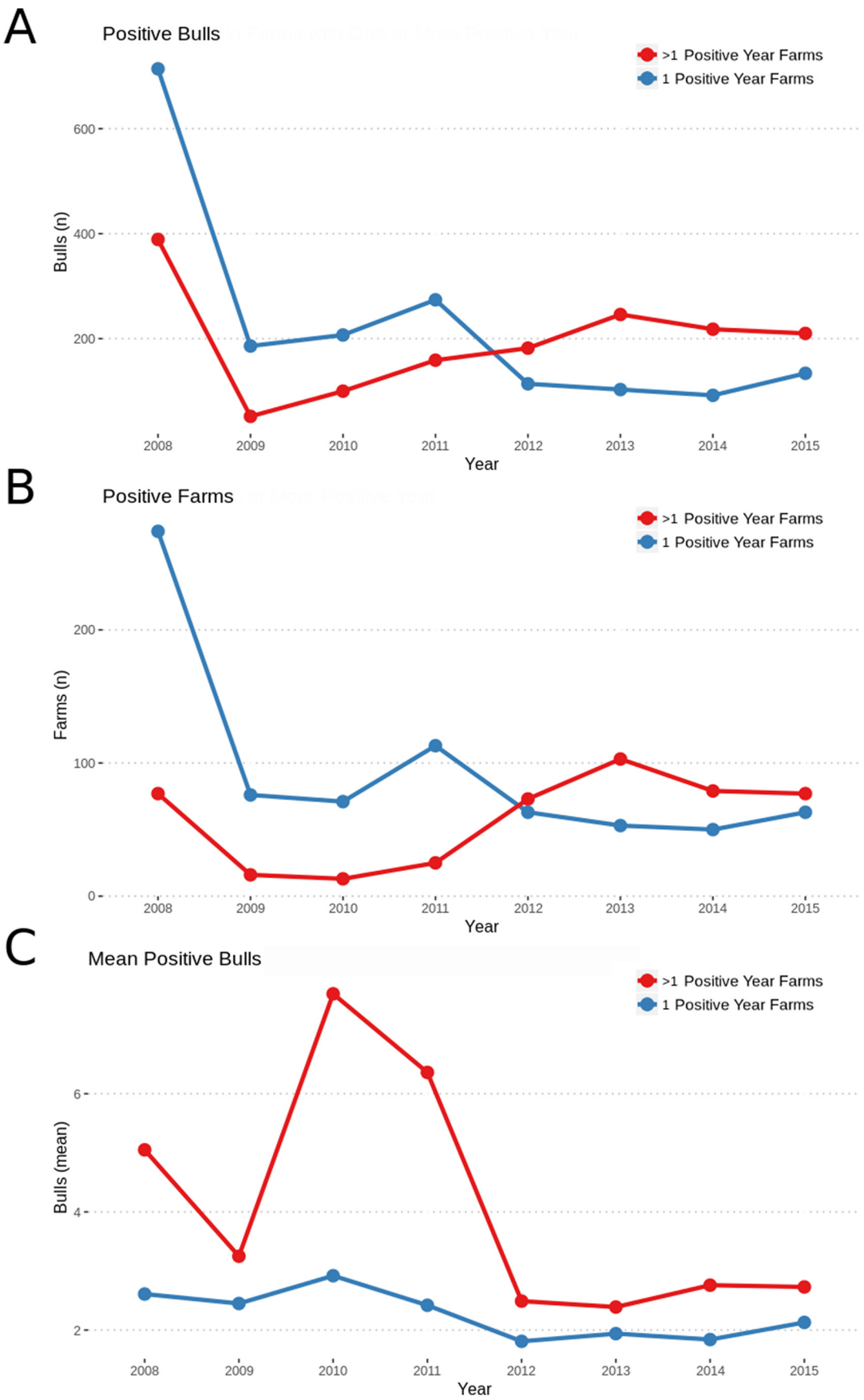

Fig. 2. Positive events registered among farms with a single or multiple positive tests for *Tritrichomonas foetus* in eight years. A. Carrier bulls in farms with one (blue line) or multiple (red line) positive registers in 8 years. B. Farms with one (blue) or more than one (red) event registered in eight years. C. The mean positive bulls per farm is shown for farms with one (blue line) or multiple (red line) positive years. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

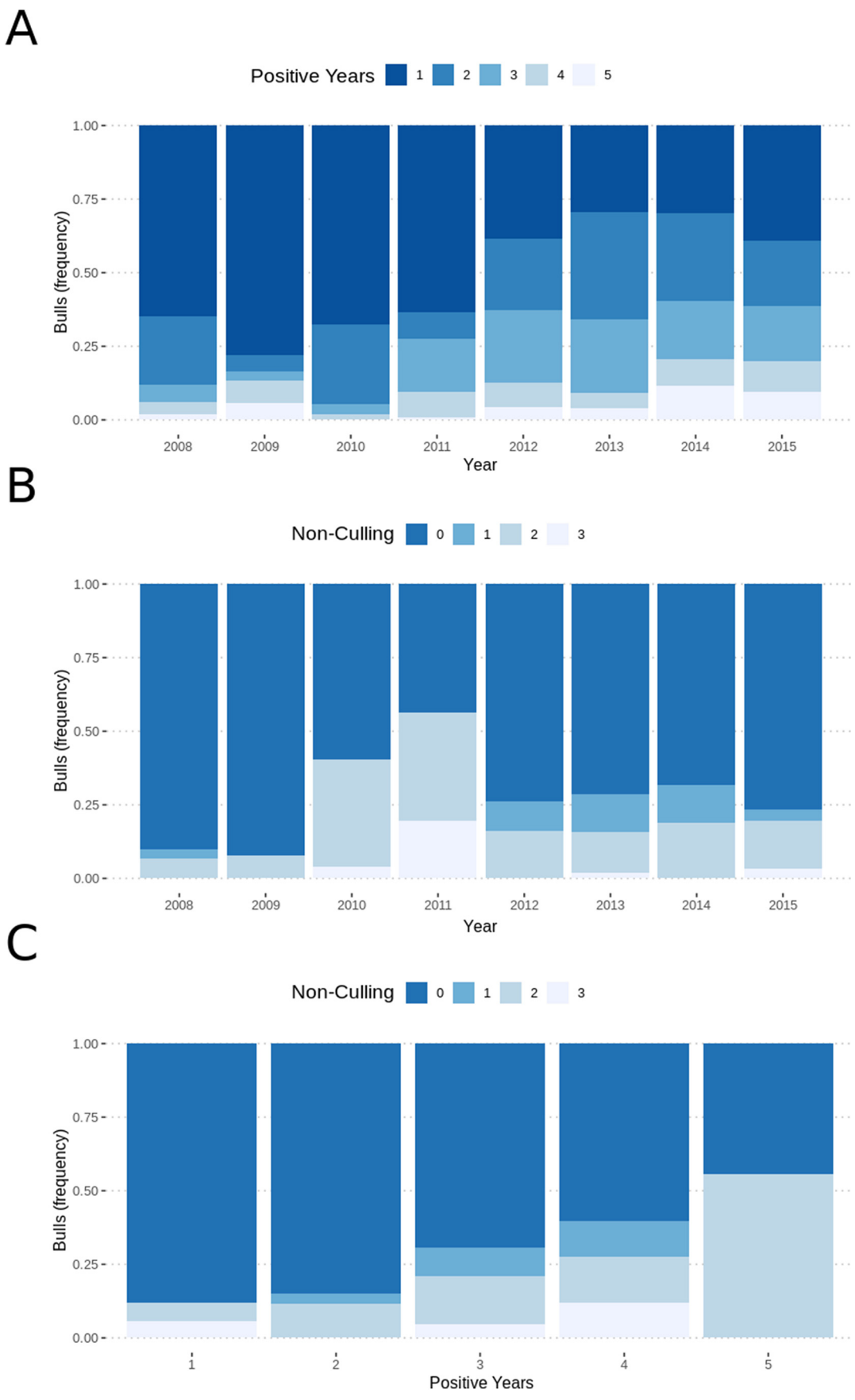

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Fig. 3. Relationship between persistence and non-culling in bovine trichomoniasis in La Pampa. A: Bulls with positive results recorded each year were segregated according to their belonging to farms with one or more years of positive tests (positive years = 1–5). B. Positive tests recorded each year are shown in non-culling categories. Category 0 includes carrier bulls detected in farms with no evidence of non-culling. Categories 1 to 3 represent carrier bulls in farms with, at least, one non-culled carrier bull. Category 1 shows carrier animal in farms that have kept infected animals under the same owner in the same farm. Category 2 contains carrier bulls in farms that have kept positive animals in the same farm under a different owner. Carrier bulls in farms that have imported infected bulls are in category 3. C. The proportion of carrier bulls from farms in different non-culling categories according to the the number of positive events recorded in different years.

identify subjects that require further evaluation.

As much as 70% of the recent cases of bovine trichomoniasis in La Pampa were here related to farms that are in non-accomplishment with the culling rule (Fig. 3). Farms that have had positive results and that did not cull or that have incorporated carrier bulls represent a significant proportion of the positive bulls in recent years.

5. Conclusion

This study showed that the recent cases of bovine trichomoniasis in La Pampa would be mostly related to the non-accomplishment with the culling rule as well as to errors in the assignation of a carrier status.

Culling *T. foetus* carrier bulls is the best known method for the control of bovine trichomoniasis. It is expected that a massive compulsory control plan could ensure the accomplishment of the best known control measure.

False positive tests should not determine the exclusion of healthy bulls. Confirmation of culture results through standard PCR procedure is advised. However, it would be more appropriate to re-test presumptive carriers through the examination of a new sample. State regulations should in this case consider the systematic revision of each positive case.

Ethical statement

The study did not involved the use of human or animal subjects or samples. Data were gently provided by MV D. Dubie from the Colegio de Veterinarios de la Provincia de La Pampa. Procedures are referenced to original works.

Declaration of Competing Interest

The author declares that there is no conflict of interest.

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