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Article

Brucella spp. seroprevalence in water buffalo *Bubalus bubalis* in the states of Campeche, Tabasco, and Oaxaca, Mexico

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Abstract:

Brucellosis is a serious zoonotic endemic disease in more than 170 regions worldwide. It is primarily a reproductive disease affecting human and animal health, and its effects can

damage producers, and regional and national economies. In Mexico, cattle are a vital protein source and a substantial portion of the livestock sector. As an alternative animal protein source, water buffalo *Bubalus bubalis* was introduced to Mexico in the early 1990s and is now grown in almost all its states. However, more than 30 yr after its introduction, minimal data is available on brucellosis epidemiology in this species. *Brucella* spp. serological frequency in water buffalo from the states of Campeche, Tabasco, and Oaxaca, Mexico, was estimated using a cross-sectional study with non-probabilistic sampling. A total of 825 animals from eighteen herds were sampled. Screening was done using the Rose Bengal card test and confirmation with a Rivanol test. Frequencies were calculated by state and municipality. Overall frequency was 4 % with the card test and 3 % with Rivanol. All three states had seropositive cases, and 66.6 % of the sampled production units had seropositive buffalo. This constitutes the first report for the states of Campeche and Oaxaca. Although *Brucella* spp. seroprevalence can change over time and responds to multiple variables, the identification of seropositive animals in an eradication zone (Campeche) is serious given the current active nationwide campaign against brucellosis.

Key words: Abortion, Brucellosis, Eradication, Epidemiology, Livestock economics.

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Introduction

Brucellosis, also known as Mediterranean fever or Malta fever, is one of the most common and serious zoonotic diseases worldwide, and is considered endemic in more than 170 regions and countries[(1)]. First described in detail in 1886[(2)], it remains a public health problem in developing countries, and is present throughout Latin America[(3)].

Bovine brucellosis, also known as “contagious abortion”, is normally caused by the bacteria *Brucella abortus*. This is a Gram-negative, facultative intracellular, obligate aerobic coccobacillus lacking a capsule. Primarily a reproductive disease, symptoms in bulls include epididymitis, orchitis, and arthritis, while in cows abortion and placental retention occur[(2,4)].

Brucellosis seriously affects cattle health, and consequently producer income. Financial losses occur as the cumulative effect of reduced milk production, higher abortion and stillbirth incidences, selective culling of infected animals, blocked animal sale and export,

wasted human labor, and medical and veterinary expenses, in conjunction with administrative and governmental expenses incurred in research and control programs, among others[5].

Over the last 30 yr, eradication programs in Mexico have tried to identify and eliminate infected animals. However, these programs are hindered by latent infections, the disease's long incubation time, the partial protection of vaccines, and the difficulty in distinguishing antibodies in vaccinated animals from those produced by natural infection[6].

Water buffalo *Bubalus* sp. is an important domestic species worldwide[6]. They are robust and can adapt to different environments and climates[7]. In *Bubalus* sp., brucellosis is caused mainly by *Brucella abortus*, which has a 9.7 % global seroprevalence in the species[1]. When infected, outward clinical symptoms (e.g. abortions) are infrequent, although infected cows commonly excrete *B. abortus* in their milk[6].

In Mexico, the Ministry of Agriculture and Rural Development (SADER) reported a *B. bubalis* population of more than 58,000 distributed in 29 of the 32 states in July 2022; populations are growing fastest in the states of Campeche, Chiapas, Tabasco, Oaxaca and Veracruz[8]. Since the species was first introduced to Mexico in the early 1990s, minimal research has been done on the epidemiology of the diseases that *B. bubalis* shares with *Bos taurus*, and *Bos indicus* and their crosses. The SADER and other federal institutions have yet to address this data shortfall; indeed, as of November 2024 *B. bubalis* is still not included in the National Campaign against Brucellosis in Animals (Mexican Official Standard NOM-041-ZOO-1995), and there is no reliable and freely accessible information on the *B. bubalis* population in Mexico. After more than 30 yr in the country, there is little information on brucellosis and its seroprevalence in *B. bubalis*. The present study objective aimed to estimate *Brucella* spp. serological frequency in water buffaloes in the states of Campeche, Tabasco and Oaxaca, Mexico.

Material and methods

Study area and design

A cross-sectional epidemiological study was done using SADER data from the states of Campeche, Tabasco and Oaxaca[8]. Buffalo production units (BPU) were selected using non-probabilistic snowball sampling in three steps: 1) The two main water buffalo breeder associations in Mexico were notified of the study and ranchers were invited to participate; 2)

A virtual presentation was given to describe the project objectives and details to interested breeders; 3) Sampling dates for each state and BPU were established in coordination with the breeders who agreed to participate. The study spanned from January 2023 to March 2024. Sampling was carried out in February 2023 in Campeche, July 2023 in Tabasco, and February 2024 in Oaxaca. A survey was applied at each participating BPU to obtain information about animal management, vaccination and deworming schedules, as well as general information. Each animal was individually identified by its SINIIGA tag number, when present, or the internal control number assigned within each BPU. Data on animal age, sex, and body condition, among other parameters, were collected.

Sample size

The total *B. bubalis* population of each state in the study (N) was calculated based on producer participation and total herd size at each participating BPU. Total population was of 2,309 animals in Campeche, 1971 in Tabasco and 190 in Oaxaca. The sample size per state was calculated using the EpiMuestra statistical program[9] for a simple random sampling with an expected 0.5% prevalence[4]; the resulting "n" were 330 in Campeche, 322 in Tabasco and 128 in Oaxaca. The number of animals sampled per BPU corresponded to its proportion of the total population in the state where it was located. Within each BPU, animals were sampled randomly and including all ages, depending on the management practices at the BPU. Final sample counts by state were 393 in Campeche, 300 in Tabasco and 132 in Oaxaca.

Biological sample collection

Blood was collected from the external jugular vein, using vacuum tubes without anticoagulant. The samples were kept at room temperature for 50 min, then refrigerated. They were centrifuged at 7,280 xg for 10 min, the serum was separated and placed in plastic aliquot containers at -28 °C until used in serological tests.

Serological tests

The Rose Bengal card test (RBT) was used for screening and Rivanol (RV) as a confirmatory test. In both tests, the sera and solutions were used at room temperature, after homogenization.

In the RBT, a suspension of inactivated *Brucella abortus* strain 1119-3 was used, buffered and stained (pH 3.6, 8 % concentration) (Lot 4720042, expiration: March, 2025). Buffered suspension (30 µL) was mixed with an equal amount of serum, the samples were mixed and kept in motion for 4 min using a rocker. Using an agglutination reader to identify the presence or absence of agglutination, test results were interpreted as suspicious or negative.

In the RV test, a heat-inactivated, colored, 4 % concentration cell package of *Brucella abortus* strain 1119-3 was used (pH 5.8 to 6.2) (Aba Test Rivanol). Rivanol solution (400 µL) was placed in an equal amount of serum in 1.5 mL microtubes, the solution mixed by inverting the microtube several times, allowed to stand at room temperature for 25 min, and centrifuged at 2,000 rpm (29 xg) for 5 min. Different amounts of supernatant (80, 40, 20, 10 and 5 µL) were placed in each quadrant of the plate from left to right (the dilutions correspond to concentrations of 1:25, 1:50, 1:100, 1:200, 1:400, respectively), mixing them with 30 µL antigen. Once mixed, the solutions were spread over an approximate diameter of 2-3 cm, the plate kept rotating to the right for 6 min and to the left for 6 min more. Using an agglutination reader, the test was interpreted as positive when agglutination occurred at concentrations ≥ 1:25. In both tests, the positive control was bovine serum previously identified and classified as strong positive (+++).

Statistical analysis

Frequencies and 95% confidence intervals were calculated with the VassarStats: Website for Statistical Computation (www.vassarstats.net).

Results

A total of 825 blood samples were analyzed with the RBT. These were collected at 18 BPU, in nine municipalities in the three states. Agglutination was identified in 33 samples (4 %). With the RV test, 25 samples (3 %) agglutinated at the 1:25 concentration (Tables 1 and 2).

Table 1: Rose Bengal (RB) and Rivanol (RV) agglutination in *B. bubalis* sera samples from three states, agglutination frequencies, and frequencies of affected production units (BPU)

States	n	RB	RV 1:25	RV 1:50	RV 1:100	RV 1:200	RV 1:400	BPU +
Campeche	393	11	8	7	6	5	4	4/7
Tabasco	300	19	14	14	11	8	5	7/9
Oaxaca	132	3	3	3	2	2	1	1/2
Total	825	33	25	24	19	15	10	12/18
Frequency, %		4.0	3.0	2.9	2.3	1.8	1.2	66.6
$CI_{95\%}$		2.8-5.6	2.0-4.5	1.9-4.3	1.4-3.6	1.0-3.0	0.06-2.2	43.7-83.7

The highest frequency of animals with antibodies at a ≥1:100 concentration was 8 % in Tabasco, and the lowest was in Oaxaca.

Table 2: Overall *Brucella* spp. seroprevalence detected by Rivanol by state

States	n	≤ 1:50	Frequency (%)	$CI_{95\%}$	≥ 1:100	Frequency (%)	$CI_{95\%}$
Campeche	393	15	3.82	2.3-6.3	15	3.8	2.3-6.3
Tabasco	300	28	9.33	6.3-13.3	24	8.0	5.3-11.8
Oaxaca	132	6	4.55	1.8-10.0	5	3.8	1.4-9
Total	825	49	5.9	4.4-7.8	44	5.3	3.9-7.1

All three states had seropositive animals, although some BPU and municipalities in each state had no positive animals (e.g. Carmen in Campeche and Macuspana in Tabasco) (Table 3).

Table 3: *Brucella* spp. seroprevalence detected by Rivanol in the studied municipalities

States	Municipalities	n	≤ 1:50	Frequency (%)	$CI_{95\%}$	≥ 1:100	Frequency (%)	$CI_{95\%}$
Campeche	Palizada	255	13	5.1	3-8.51	12	4.7	2.7-8.0
	Escárcega	11	2	18.1	5.1-47.7	3	27.2	9.7-57.5
	Carmen	127	0	-	-	0	-	-
Tabasco	Centro	198	24	12.1	8.2-17.4	23	11.6	7.8-16.8
	Nacajuca	15	0	-	-	0	-	-
	Huimanguillo	45	4	8.8	3.5-20.7	1	2.2	3.9-11.56
Oaxaca	Macuspana	42	0	-	-	0	-	-
	San Pedro Mixtepec	31	0	-	-	0	-	-
	Pinotepa Nacional	101	6	5.9	2.7-12.3	5	4.9	2.1-11.7
Total		825	49	5.9	4.4-7.8	44	5.3	3.9-7.1

Out of the 93 bulls and 723 cows sampled, no bulls were positive. Among the cows, 33 exhibited agglutinations in the RBT and 25 in the RV test (1:25). Of the latter, only 10 exhibited agglutinations at 1:400, which differed from the higher concentration (χ^2= 7.4; *P*=0.025).

Sampled animal age ranged from one month to 22 yr. Five animals less than one year old showed agglutination with the RBT, and four of these exhibited antibodies in the RV test. In Campeche, a 6-mo-old calf exhibited agglutination at 1:400 (BPU 7). In Tabasco, three calves (all 7-mo-old) exhibited antibodies, and one had antibodies at 1:400 (BPU 10).

A total of 24 animals >1 yr and <10 yr were seropositive in the RBT, of which 20 also had agglutination in the RV test: 4 in Campeche (BPUs 1, 2, 6 and 7); 13 in Tabasco (BPU 10 and 16); and 3 in Oaxaca (BPU 16). Antibody concentrations were from 1:25 to 1:400.

Four animals >10 yr exhibited agglutination with the RBT, and two (both from Campeche) had antibodies in the RV test (Table 4). Of these two, one (20 yr old, BPU 1) had antibodies that agglutinated at 1:50, and the other (12 yr old, BPUI 7) at 1:25. Results did not differ between age groups (χ^2= 14.4, *P*= 0.702).

Table 4: Agglutination by the RB and RV tests in *B. bubalis* serum samples by age group

Age (months)	n	RB	RV 1:25	RV 1:50	RV 1:100	RV 1:200	RV 1:400
≤ 12	204	5	4	4	3	2	2
13 – 120	525	24	19	19	16	13	8
≥121	96	4	2	1	0	0	0
Total	825	33	25	24	19	15	10

Discussion

The limited research on *Brucella* spp. in *B. bubalis* in Mexico shows prevalences higher than in cattle. In a study of three herds in the south of the state of Veracruz, seroprevalence in *B. bubalis* was 13 % by RBT and 7 % by RV[10]. In contrast, the National Agricultural Health, Safety and Quality Service (SENASICA), for 2017, reported a total of 8,119 positive tests in cattle of a total of 3,312, and 166 tests (0.24 %)[11], based on established quarantines, a 0.22 % national frequency[12].

In 2022, a second study was published of three BPU in Veracruz and two in Tabasco, with a 0.5 % prevalence using the ELISA test[4]. This is comparable to the 0.05 % national prevalence in cattle reported for 2023[13]. Both are well below the pre-2010 worldwide seroprevalence in *B. bubalis* of 20.8 %, and even below the 4.2 % reported for 2010 to 2020[1]. This notable reduction has been attributed to on-site control measures proposed by the World Organization for Animal Health (WOAH), which has acknowledged improved control of zoonotic diseases in developing countries[1]. The two studies done in Mexico also show a reduction in prevalence. However, they only address southern Veracruz and northern Tabasco, both known for having relatively large water buffalo populations. Given that official data report *B. bubalis* populations in 29 states[8], many more states and regions need to be sampled to produce a more complete picture of *B. bubalis* epidemiology, particularly in terms of serious potential zoonoses such as brucellosis[14].

Contrasting the present results with research from other parts of the world helps to contextualize it. Public policy and animal health guidelines in countries that have controlled brucellosis (e.g., Canada, Japan, Australia and New Zealand)[15] can serve as models. However, even countries with zero *Brucella* spp. seroprevalence[16] continue to struggle with poor livestock performance production and animal health due to other diseases. This suggests the necessity of a broad perspective including other diseases in the search for comprehensive solutions, with a clear priority for zoonoses[1].

Seropositive animals were detected in all three states; this confirms previous findings for Tabasco but constitutes a first report for Campeche and Oaxaca. This is noteworthy because the state of Campeche is currently in the eradication phase[17]. The BPU in Palizada and Escárcega municipalities had animals seropositive with RV, four of them at 1:400. The municipality of Carmen had no seropositive animals. The states of Tabasco and northeast Oaxaca, where the studied BPU are located, are both currently in the control phase[17]. All three states had BPUs without seropositive animals. It is important to remember that the sampled BPU are only a sample of each state's total *B. bubalis* population.

Interactions of humans and animals with the environment can increase or decrease disease contagion risk factors in each ecological niche or endemic area[18]. A recent study proposes rainfall as a determining factor in increased seroprevalence in water buffaloes in Mexico for *Neospora caninum*, *Toxoplasma gondii* and *B. abortus*[4]. This may partially explain differences in seroprevalences between the studied states. Climate in Oaxaca is drier (Aw, warm subhumid with summer rains) than in Tabasco (Af, warm humid with rains year round) and Campeche (Am, warm humid with summer rains)[19]. The differences in seroprevalences between municipalities, and even BPU location, may also respond to climate factors. For example, the municipality of Carmen, Campeche, contains all three climate types described above[20] but no seropositive animals were detected. In Oaxaca, both studied municipalities and the BPUs have the same climatic characteristics[21,22]; only three animals were detected as seropositive by RV (one at 1:400), all in Pinotepa Nacional municipality.

Average animal age was 7.21 yr in Campeche, 3.38 in Tabasco, and 4.59 in Oaxaca. Maximum age was 22 yr and the minimum was one month. In *B. bubalis*, age is an important risk factor which increases year on year for parasitic diseases such as neosporosis and toxoplasmosis[4]. This behavior has been recorded in *B. bubalis* for bacterial diseases such as leptospirosis and viral diseases such as herpesvirus[23]. The presence of maternal antibodies in young animals raises the question of vaccination. Application of brucellosis vaccines was reported in three (17 %) of the 18 studied BPU, although the last application was in 2017.

In *B. bubalis*, ecto- and endoparasite control measures, as well as food supplementation and vaccination plans, are less strictly applied than in cattle, partially because water buffalo are thought to have higher innate resistance[24]. In contrast, water buffalo often live in proximity to communal drinking troughs for other domestic and wild species, causing the possibility of contact with a large number of pathogens to be higher than in cattle or other livestock[25]. The above factors, weather conditions, and individual herd management practices, are probably responsible for the different seroprevalences observed here in the states and BPU[3,24,25].

Brucellosis continues to be a public and animal health challenge in Mexico. There are clear risk factors for animals and humans, the regulatory environment is complicated, and culling infected animals poses serious economic hardship. In addition, the monitoring programs of

State Livestock Development Committees have a very short reach, even thirty years after publication of relevant federal regulations (NOM-041-ZOO-1995 and NOM-022-SSA2-1994)[1,3,26]. The detection of a 3 % seroprevalence with RV (1:25) in all three studied states is alarming, but confirms that Mexico has one of the highest brucellosis prevalences in the Caribbean and Central America[26]. Interpretation of the present results requires caution since they constitute merely a sample of just three of Mexico's 32 states, seroprevalence can vary in response to the particular characteristics of each BPU, region, state and country, and can vary over time[1].

Limitations in the present study include lack of a database with public access and updated information on the *B. bubalis* population in Mexico. Convenience sampling can create bias; for example, BPU that decided not to participate in the study and those that were not aware of it prevented broader data collection, possibly masking the real situation of the total population. The absence of production or preventive medicine records in most of the BPU made data analysis particularly challenging, which may have affected the results. The RB and RV tests are useful diagnostic assays, but other techniques are needed to enrich the data on *B. bubalis* and other livestock. Techniques such as immunofluorescence, ELISA, radial immunodiffusion with native hapten, histopathology, PCR, and field strain isolation, among others, would undoubtedly strengthen the database.

Conclusions and implications

Although water buffalo's apparent resistance to brucellosis suggests production advantages and opportunities, the species' epidemiological status needs to be more clearly defined. New diagnostic, bacterial isolation and analysis techniques need to be developed, and *B. bubalis* should be incorporated into relevant federal programs. New research is also needed to control brucellosis and other diseases that water buffaloes share with cattle as well as other domestic and wild animals.

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Conflict of interest

The authors declare no conflict of interest.

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