Seroprevalence study of the main causes of abortion in dairy cattle in Morocco

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Abortion,
Cattle,
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Summary
Sera from 221 cattle were collected in 25 farms in Morocco to investigate the evidence and circulation of some of the main bovine abortive agents in the dairy cattle farming, where abortions are often reported. All sera were examined for brucellosis, 176 for neosporosis, 88 for leptospirosis, and 42 for Bovine viral diarrhoea (BVD/MD), Bovine Herpesvirus 1 (BHV-1) (Infectious bovine rhinotracheitis, IBR/IPV), and Bovine Herpesvirus 4 (BHV-4) infections (at least 1 sample per herd). Abortions were reported in 23 (10.4%) of the 221 tested cattle. Antibodies against the investigated pathogens were detected in all samples tested, with an overall seroprevalence of 33.48% for *Brucella*, 9.09% for *Leptospira*, 8.52% for *Neospora*, 37.71% for BVDV, 50% for BHV-1, 9.52% for BHV-4. As for *Leptospira* antibodies against serovars Hardjo, Pomona, and Tarassovi were identified. Mixed infections were common. The lack of evidence of non-infectious factors epidemiologically related to abortions suggested that the investigated agents are to be considered important risk factors in the dynamic of the abortion syndrome, even if further investigations are necessary to identify the abortion cause. Particular attention should be paid on brucellosis, considering the high seroprevalence and its zoonotic relevance.

Indagine sierologica sulle principali cause di aborto nell'allevamento del bovino da latte in Marocco

Parole chiave
Aborto,
Bovino,
Marocco,
Sierologia.

Riassunto
La presenza di aborti è un evento riportato di frequente nell'allevamento del bovino da latte in Marocco, sono tuttavia disponibili poche informazioni sugli agenti infettivi potenzialmente responsabili di tale fenomeno. Al fine di indagare la presenza e la circolazione dei principali agenti infettivi che causano aborto nel bovino sono stati raccolti 221 sieri provenienti da 25 allevamenti di bovine da latte. Tutti i campioni sono stati testati sierologicamente per brucellosi, 176 per neosporosi, 88 per leptospirosi, e 42 per la Diarrea virale bovina (BVD/MD), e infezioni da herpesvirus bovino tipo 1 (BHV-1, IBR/IPV) e da BHV-4 (almeno un campione per allevamento). Dei 221 bovini campionati, 23 (10,4%) risultavano aver abortito nell'ultimo anno. Sono state rilevate positività verso tutti i patogeni indagati con valori complessivi di sieroprevalenza del 33,48% per *Brucella*, 9,09% per *Leptospira*, 8,52% per *Neospora*, 37,71% per BVDV, 50% per BHV-1, 9,52% per BHV-4 e con presenza diffusa di infezioni miste. Per *Leptospira* nello specifico sono state trovate positività sierologiche verso le sierovarianti Hardjo, Pomona e Tarassovi. Nessun fattore non-infettivo è risultato essere epidemiologicamente correlabile ai casi di aborto. I risultati ottenuti indicano che gli agenti infettivi indagati circolano nella regione e rappresentano importanti fattori di rischio nello sviluppo di sindromi abortigene; ulteriori indagini andranno focalizzate sull'identificazione diretta delle cause di aborto. Particolare attenzione andrebbe rivolta allo studio della brucellosi, data l'elevata sieroprevalenza riscontrata nel bovino e le possibili implicazioni zoonosiche.

Introduction

The Moroccan cattle farming system has known a significant intensification since the Dairy Plan was launched in the 1970s, with the aim of meeting the increasing demand of milk and dairy products to the benefit of a rapidly growing population (FAO 2011, Sraïri *et al.* 2011). Yet, smallholder farms still constitute more than 80% of the farming sector and poor hygiene conditions are still widely prevailing (Sraïri *et al.* 2009). Reproductive failure due to abortion is a critical issue in cattle farming because of economic losses and public health implications. However, only scarce and sparse information is available about the situation regarding cattle abortion in Morocco and the role of infectious abortions in reproductive failure. Brucellosis being the only exeption, as it has been investigated in 3 national surveys carried out in 1975, 1989, and 1994 (Belkhayat 1976, FAO 1997). The aim of the present study was to investigate the evidence and circulation of some of the main bovine abortive agents (Holler 2012) in Morocco through a sero-epidemiological survey focused on brucellosis, leptospirosis, neosporosis, Bovine viral diarrhoea (BVD/MD), Bovine Herpesvirus 1 (Infectious bovine rhinotracheitis, IBR/IPV), and Bovine Herpesvirus 4 (BHV-4) infection.

Material and methods

Context

The study was carried out in El-Gharb, an irrigated region located in the Northwest of Morocco. The fertile plain of El-Gharb is largely devoted to agriculture and dairy cattle production and the local semi-intensive farming system can be considered representative of the dairy sector in Morocco. Smallholder farms still constitute the majority of production facilities, as much as in the other irrigated schemes (FAO 2011), even if some medium scale farms are present. The farm management involves the widespread use of artificial insemination, the calf separation from the mother after the birth, and the presence of a dedicated milking room. Cattle are usually raised in fenced areas during the day and kept in boxes during night. The bulls live separately, but the natural insemination practice is still common when more than 2 artificial inseminations lead to reproductive failure. Veterinary services, including advice and health care, are provided by private practitioners holding a sanitary mandate and are assisted by Government veterinarians in case of compulsory notifiable diseases.

Sampling and data collection

The seroprevalence of the abortive agents was assessed by collecting 221 cattle sera in 25 farms. The selected targets were cows and heifers more than 18 months of age and with or without a history of individual abortion. The sampling plan foresaw the following scheme: 10 sera from herds with more than 11 animals; sera from all the subjects in herds with less than or equal to 11 animals. The number of 10 animals per herd was considered adequate to detect infections circulating in the small-scale farm system characteristic of the region. Such a non-probability methodology is widely adopted when the aim is to assess disease prevalence at herd level (Rodolakis *et al.* 2007). Furthermore, only 7 of the 25 selected herds had more than 11 cattle, only 1 herd counted more than 50 cattle for an overall amount of 352 animals. Thanks to the farmers' collaboration, a questionnaire could be filled out: epidemiological and clinical data related to abortion events were collected during the farm visit to better assess the role of the investigated agents in the etio-pathogenesis of the abortion cases. Sampling and data collection were performed in a single visit. The serum samples were collected using sterile 10 ml evacuated glass tubes from the jugular vein and transported at 4°C to the laboratory for testing.

Laboratory analyses

At the laboratory, the sera were separated by centrifugation of blood at 3,000 x g for 10 minutes, transferred in sterile tubes, identified and stored at -20°C until tested. Serological tests were performed to investigate the presence of specific antibodies against *Brucella*, *Leptospira* with special emphasis on serovar Hardjo, viral agents (BVDV, BHV-1, BHV-4) and *Neospora caninum*.

To assess the seroprevalence of bovine brucellosis the sera were examined by Rose Bengal Test (RBT) (Veterinary Laboratory Agency – VLA, Weybridge UK) according to a modified version of the OIE recommended protocol (Blasco 1994). This test is reccomended by the OIE as a suitable screening test at individual and herd level (OIE 2008). A volume of 25-30 µl of antigen (*Brucella abortus* suspension stained with Rose Bengal and buffered) was mixed on a plastic plane to ca. 75 µl of test serum and gently agitated by rotating the plate for 4 minutes; any grade of agglutination was considered as a positive reaction and positive and negative controls provided by the supplier were included in the test.

The presence of anti-*Leptospira* antibodies was assessed by indirect ELISA and Microscopic Agglutination Test (MAT). Indirect ELISA (PrioCHECK® *L. hardjo* Ab, Prionics, Lelystad, Switzerland) was used for detecting antibodies against *Leptospira* serovar Hardjo, known to be one of the most relevant serovars causing abortion in cattle and for which

cattle represent the primary maintenance host (Ellis 1994, Goomes 2006). The MAT is the serological standard test for diagnosing leptospirosis (OIE 2008). This testing method is based on the observation of microagglutination due to the presence of serovar-specific antibodies in the tested serum; it requires a dark field microscope and live antigens. The MAT was conducted using a panel of 9 strains of the main pathogenic serovars of *Leptospira* existing in the Mediterranean area (Cerri *et al.* 2003); these are: Icterohaemorrhagiae, Canicola, Copenagheni, Pomona, Hardjo, Bratislava, Tarassovi, Grippotyphosa and Ballum. Antigens were 4-8 day-old cultures of the 9 strains grown at 30°C in liquid Ellinghausen McCullough Johnson Harris (EMJH) medium. Sera were diluted in physiological solution 1:50 and mixed in the wells of a microtitrer plate with an equal volume (100 µl) of each antigen for a final screening dilution of 1:100. The occurring of a positive reaction (agglutination of at least 50% of leptospires) was verified at the microscope after 2 hours of incubation at 37°C. Two-fold serial dilutions of the positive samples were prepared and tested with the same screening procedure to obtain the titre. The test included a positive control, a negative control, and an antigen control for each serovar.

Indirect ELISA kits Abortion screen (Cypress Diagnostics, Hulshout, Belgium) and ID Screen® *Neospora caninum* indirect Multi-species (ID-vet, Montepellier, France) were used to detect antibodies anti-BVDV, anti-BHV-1, anti-BHV-4 on the one hand and anti-*N. caninum* on the other hand. All ELISA kits included in the study were performed according to the manufacturers' recommendations. Due to limited resources, not all samples could be tested with all the described diagnostic procedures and priority was given to assess the seroprevalence of *Brucella*, *Leptospira* and *Neospora caninum*, rather than viral diseases. Consequently, 221 sera were tested for brucellosis, 176 for neosporosis, 88 for leptospirosis, and 42 for viral infections (at least 1 sample per herd).

Results

The questionnaire provided the information about the occurrence of abortion: cases of abortion were reported in 23 (10.4%) of the 221 tested cattle; 12 (48%) among 25 herds had experienced abortions, 4 of them (16%) had more than 1 case, and the abortion rate varied from 1.35% to 36% per herd with an average of 12%. The history of the cases of abortion did not show any evident connection with non-infectious causes throughout the calving season. None of the investigated herds was subjected to any vaccination programme. The serological results are summarized in Table I. Antibodies against all the investigated pathogens were detected, with an overall seroprevalence varying from 8.52% to 50%. In particular, for *Brucella*, BVDV, and BHV-1,

Table I. *Infectious causes of abortion in dairy farms of Morocco.*

Infectious agent	N° positive herds/ tested herd	N° positive herds with abortion	N° tested samples	N° positive samples and prevalence	N° positive samples with abortion	N° samples with mixed infections
Brucella	22/25	10	221	74 (33.48%)	10	16 (7.24%)
Leptospira	5/18	4	88	8 (9.09%)	1	2 (2.27%)
Neospora caninum	10/25	6	176	15 (8.52%)	3	7 (3.98%)
BVDV	12/25	6	42	15 (37.71%)	8	14 (33.33%)
BHV-1	12/25	7	42	21 (50%)	11	15 (35.71%)
BHV-4	4/25	2	42	4 (9.52%)	2	3 (7.14%)

Table II. *Mixed infections in Morocco dairy cattle.*

	Brucella	*Leptospira*	*N. caninum*	BVDV	BHV-1	BHV-4
Brucella	-	0	3	10	10	1
Leptospira		-	2	0	0	0
N. caninum			-	2	3	0
BVDV				-	9	2
BHV-1					-	2
BHV-4						-

Table III. *Mixed infections in Morocco dairy cattle with abortion.*

	Brucella	*Leptospira*	*N. caninum*	BVDV	BHV-1	BHV-4
Brucella	-	0	1	5	6	0
Leptospira		-	0	0	0	0
N. caninum			-	1	1	0
BVDV				-	4	1
BHV-1					-	1
BHV-4						-

Table IV. *Presence of infectious agents and abortions in Morocco dairy farms.*

N° abortions	N° herds	*Brucella*	*Leptospira*	*N. caninum*	BVDV	BHV-1	BHV-4
0	13	12	1	4	5	6	2
1	8	6	3	5	4	4	0
>1	4	4	1	1	3	3	2

Table V. Leptospira *species in Morocco dairy farms according to MAT results.*

Positive herds	Ic	Ca	Cop	Po	Har	Bra	Tar	Gri	Bal
Farm 1	-	-	-	-	1	-	-	-	-
Farm 3	-	-	-	3	-	-	-	-	-
Farm 5	-	-	-	2	-	-	-	-	-
Farm 7	-	-	-	1	-	-	-	-	-
Farm 10	-	-	-	-	-	-	1	-	-

Ic = Icterohaemorrhagiae; Ca = Canicola; Cop = Copenagheni; Po = Pomona; Har = Hardjo; Bra = Bratislava; Tar = Tarassovi; Gri = Grippotyphosa; Bal = Ballum.

the seroprevalence was greater than 30%. With regard to positive farms, the percentage of positive samples varied from 1.13% for *Leptospira* spp. to 100% for *Brucella*, recorded in a herd with cases of abortions. Furthermore, *Brucella* was the pathogen with the highest seroprevalence, with 22 positive herds (10 with cases of abortions) among which 7 had a percentage of positive samples greater than 50%. In 19 farms (10 with cases of abortions), sera showed antibodies against more than 1 abortive agent, suggesting the presence of mixed infections (Tables II and III). Numerous animals had antibodies against both *Brucella* and BVDV or BHV-1 and in some cases this evidence coincided with cases of abortion. To better assess the distribution of positive herds and abortions, 3 groups of herds were identified according to the abortion occurrence: no abortion, 1 abortion, more than 1 abortion (Table IV). The results confirmed that the infections were uniformely distributed in the herds despite the presence of abortion. Regarding *Leptospira* analyses, the MAT provided the data about serovars' circulation (Table V). Eight sera were positive to the MAT: 1 against Hardjo with titre of 1:800, 6 against Pomona with titre ranging from 1:100 to 1:400, 1 against Tarassovi with titre of 1:100. The Hardjo positive animal was confirmed by ELISA and it was associated with an abortion case.

Discussion

Brucellosis, leptospirosis, neosporosis, BVD, IBR, and BHV-4 infection represent some of the most common causes of infectious abortion worldwide (Holler 2012). For this reason, they are usually included in the routine serology on aborting dams (Anderson 2007) and they were the selected diseases for investigation in our survey.

This survey reveals a widespread circulation of the investigated abortive agents in the Moroccan dairy farming system. Considering the empiric sampling plan, an intra-herd evaluation seems to be premature due to the small samples' size included in some tests and that is particularly true for viral infections. However, some considerations are possible on the basis of the overall seroprevalences results. Information on brucellosis is the most complete and detailed, the sampling plan for brucellosis was completely fulfilled in this study.

Brucellosis is nowadays quite rare in countries that are carrying out eradication programs, but it is still a primary matter of concern in developing countries (Godfroid *et al.* 2005). The Mediterranean region and Middle East are high prevalence areas where brucellosis has been recognized from the 1970s to be a widespread zoonotic disease (Refai 2002). Recent works report a seroprevalence in cattle of 26.3% in Algeria (Aggad & Boukraa 2006) and 18.52% in Egypt (Samaha *et al.* 2009) using RBT. In Morocco a serological survey was conducted in 1974 on 20,000 dairy cattle leading to the identification of the Casablanca, Rabat, Sale, Fes, and Meknes region as the areas with the highest level of infection with a seroprevalence up to 27% (Belkhayat 1976). This first investigation was followed by 2 other surveys carried out in 1989 and 1994 (FAO 1997). *Brucella abortus* was isolated in 1982 from abortions in a herd of 200 cattle (Johnson *et al.* 1984) and *B. abortus* strains isolated from aborted calves were characterized as biovars 1 and 3 in a further study (Taoudi *et al.* 1984).

The datum on *Brucella* overall seroprevalence reported in this survey seems to be remarkably high (33.48%) compared to other surveys performed in North Africa and even higher than the data of the 1976 survey in Morocco. However, the fraudulent use of vaccine cannot be excluded given that the vaccination is practiced in the country, although subject to special authorization. Rose Bengal Test cannot distinguish among antibodies response to different *Brucella* species, so bacterial isolation has to be performed to identify which species are circulating and which are epidemiologically relevant in the region. Considering *B. abortus* and *Brucella melitensis* as the most common causes of bovine brucellosis (Refai 2002), the role of these 2 species in cattle in Morocco needs to be clarified. The presence of *B. abortus* in Moroccan cattle farms and its association with abortion were proved by

previous studies, but *B. melitensis* can also cause reproductive disorder in cow, although it is more frequent in sheep and goat. Indeed, *B. melitensis* was found associated with abortions in some Middle Eastern countries, where *B. abortus* is rare or absent (Benkirane 2006). The promiscuity among cattle and *B. melitensis* infected goats and sheep should be considered as a relevant risk factor related to the occurrence of cattle abortion in some regions.

The data about *Leptospira* (9.09%) and *Neospora* (8.52%) confirm the infections but with a low seroprevalence compared to other surveys performed in North Africa and Mediterranean region.

Several studies report variable seroprevalence values for *Leptospira* in Mediterranean cattle: 15.3% in Portugal (Rocha 1998), 8% and 18.33% in different regions of Spain (Alonso-Andicoberry *et al.* 2001, Guitián *et al.* 2001), and 0.48% in Italy (Cerri *et al.* 2003). In North Africa, a serological survey on domestic animals was performed in Egypt, including 9 cattle among which 4 resulted positive to MAT. In Morocco a recent study reported a seroprevalence of 15% in cattle and Ballum, Sejroe, and Bratislava were detected as the most prevalent serovars (Benkirane *et al.* forthcoming). The seroprevalence of neosporosis is high in many countries and it has been demonstrated that seropositive cows are more likely to abort than seronegative cows (Dubey *et al.*, 2007). In Algeria, a seroprevalence of 19.64% (Ghalmi *et al.* 2012) was observed and comparable data have been reported in Egypt, 20.43% (Ibrahim *et al.* 2009) and Senegal, 17.9% (Kamga-Waladjo *et al.* 2010). Higher and lower values were reported in Spain, 35.9% (Quintanilla-Gozalo *et al.* 1999), and Italy 11% (Otranto *et al.* 2003), respectively. The prevalence of neosporosis in Morocco is unknown, but a seroprevalence of 22% was found in El-Gharb and Meknès regions (Bouhou 2006 personal communication).

Considering the *Leptospira* data, the finding of a high titre against serovar Hardjo in an aborting cow with no other positivity supports the hypothesis of a *Leptospira* implication as possible cause of abortion, as it is well known for serovar Hardjo worldwide (Ellis 1994). A positive titre towards *Leptospira* is reported to be associated with an increased probability of abortion (Hässig & Lubsen 1998), yet the role of Hardjo infection in 1 abortion case of the Farm was not confirmed and the circulation of the serovar should be further analysed considering the single positive result found.

Serovar Hardjo has been found in a specific study focused on *Leptospira* and performed in 2013 in the same area (A. Benkirane, personal communication). This study used a lower MAT cut-off (1:20) and revealed a wider circulation of this serovar. Low titres are possibly due to old contacts with the bacteria or chronic carrier status (OIE 2008), which cannot be easily identified using a MAT cut-off of 1:100. This suggests that the problem could be underestimated and needs more attention considering the zoonotic role of Hardjo reported in some studies (Shenberg *et al.* 1977, Belmaker *et al.* 2004). The samples positive to serovars Pomona and Tarassovi prove the animals' exposure to the agents. The lack of evidence of abortion does not allow for taking epidemiological conclusions, but the presence of the 2 serovars in the area was confirmed. Abortion in cattle is known to be possibly associated to serovar Pomona infection (Anderson 2007), while the association between Tarassovi infection and abortion has not been already proved, although this serovar were identified as relevant in some cases (Hesterberg *et al.* 2009).

The *Leptospira* overall seroprevalence (9.09%) could be also influenced by the MAT panel composition. An enlarged panel could identify the circulation of relevant serovars not included in this study and recognize the occurrence of cross-reactivity due to the circulation of serovars not evaluated and still belonging to the same serogroups of the current MAT panel serovars.

Bovine viral diarrhoea virus, BHV-1, and BHV-4 are common viral infections linked to bovine abortion (Holler 2012). Numerous references are available about the seroprevalence of BHV-1 and BVDV worldwide, reporting variable but commonly high values depending on the control measures practiced. The only published data about Morocco are dated 1984 reporting a seroprevalence of 48.5% for BVDV and 62.8% for BHV-1 on 524 cattle from different areas (Mahin *et al.* 1985). Yet, the BVDV-abortion association is the topic of some on-going work that will provide updated information. The BHV-4 is considered an opportunistic viral pathogen and its role in abortion is difficult to determine (Holler 2012), its increasing importance in cattle reproduction is reported by some authors who identified the BHV-4 infection as the third most significant viral cause of bovine abortion (Kirkbride 1992, Holler 2012).

The small sample size (at least 1 sample per herd) could has been a limit in viral disease analyses, but it was sufficient to find an overall seroprevalence up to 37.71% for BVDV and 50% for BHV-1, suggesting an endemic presence of the infections and a possible high-level virus circulation, as it was found in 1984. The results reported in this paper represent the first data about BHV-4 seroprevalence in Morocco.

An average abortion rate of 12% seems to be lower than previous data reporting values up to 20% (Bouhou 2006 personal communication, Awçalla 2007 personal communication), but it is still a critical value since abortion rate higher than 5%-8% is deemed unacceptable in cattle farming

(Holler 2012). A clear relation between infection and abortion cannot be identified on the basis of a single serological test, that can only reveal the agent exposure without differentiate between recent and old infections. Acute and convalescent serum samples proving an increasing titre as well as the direct diagnosis of the abortive agent in the foetus are needed, at the least, to make a certain diagnosis (Anderson 2007).

The recurring presence of mixed infections in abortive and non-abortive dams makes the abortion cause identification even more difficult in this survey. However, the high seroprevalences found, in particular for *Brucella* and viral infections, without evidence of other non-infectious factors epidemiologically related to abortion, suggest that the investigated agents are to be considered important risk factors in the dynamics of the abortion syndrome. Further studies will be necessary to better assess the relation of the investigated agents to reproductive disorders in Moroccan dairy farms.

Among the investigated agents, *Leptospira* and *Brucella* represent 2 of the most widespread bacteria causing zoonotic diseases in the world. The evaluation of the potential zoonotic impact of bovine brucellosis and leptospirosis in Morocco should be analysed following a 'one health' approach. In particular, the high seroprevalence of *Brucella* suggests the presence of an alarming situation in bovine that needs to be better investigated not only due to the role of brucellosis on dairy farm management but also because of its impact on public health. Although the disease is notifiable, there are no available data on human brucellosis in Morocco in the latest years. Still, the high bacterial circulation in cattle population represents an important risk factor considering the poor hygiene conditions at farm level and the common consumption of raw milk and milk products in the region. Collecting information on humans will allow for achieving a better awareness on the epidemiology of brucellosis and medical and veterinarian authority collaboration is desirable to schedule an appropriate control programme.

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