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Serological data indicate a widespread presence of rabbit haemorrhagic disease in rabbit farms in Algeria

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The Algerian government has recently supported plans to develop and increase commercial rabbit farming. A necessary condition for their success is to ensure rabbits' health and protect farms from infectious diseases. Among these, Rabbit Haemorrhagic Disease (RHD) is one of the worst, causing high mortality and, thus, severe economic losses. Considering RHD's high diffusibility, accurate surveillance systems and the proper and extensive use of vaccinal prevention are paramount in protecting rabbit populations. A sero-epidemiological survey on RHD was conducted in 19 herds located in different regions of Algeria to obtain a first overview of the monitoring system's ability to detect RHD and estimate its presence and distribution in the country. The results showed that RHD is widespread in Algeria, far more than assumed based on the number of reported and diagnosed disease outbreaks. As in the rest of the world, RHDV2 was by far the prevalent, if not the only, agent of RHD in Algeria. By verifying the outcomes and results of using RHD vaccine in farms, it was shown the need to improve vaccination plans, likely through the strict application of the guidelines for RHD direct prophylaxis provided by the EU Lagmed project.

Keywords Algeria, Rabbit haemorrhagic disease, Serology, Epidemiology, Vaccination

The Rabbit Haemorrhagic Disease (RHD) virus is considered one of the most severe pathogens that considerably impacts rabbit health, causing a lethal hepatitis. RHD's high diffusibility, infectivity, and mortality rate led the World Organization for Animal Health (WOAH) to include it in the list of notifiable diseases. Its etiological agent (RHDV) belongs to the Lagovirus genus within the Caliciviridae family[1]. Since the first detection of RHDV in 1984 in China[2], a single known virulent serotype (RHDV) in rabbits has existed, and sanitary measures and vaccination have controlled it.

Nevertheless, in 2010, a new lagovirus, named RHDV type 2 (RHDV2), emerged in France with three main distinguishing features: (i) the capacity to cause RHD even in young rabbits, (ii) its different antigenic profile, and (iii) although the rabbit remains the primary host, the capability to infect and induce disease in several lagomorph species other than European rabbit[3–9]. Due to these phenotypic characteristics, nowadays, RHDV2 is the primary cause of RHD in domestic rabbits and wild animals, replacing the original "classical" RHDV. It has also become endemic in North and Central America[9–14] and, more recently, South Africa[15].

RHD is almost always fatal in naïve rabbits, but the few that survive then develop lasting systemic immunity. Both RHDV and RHDV2 induce specific antibody production usually measured by competitive ELISA (Enzyme-Linked Immunosorbent Assay) developed for laboratory diagnosis[16–19]. In surviving rabbits infected by RHDV or RHDV2, a protective systemic humoral immunity is induced through high titres of IgM and IgA detected within 3–4 days post-infection, followed by IgG after 7–8 days. At the same time, a transient, approximately two-month-long mucosal immunity is mainly based on specific IgA[17]. Conversely, in vaccinated rabbits, only IgG is usually detected with titres 10–30 times lower than those found in RHD convalescent rabbits[19] (Capucci L, Cooke B, Lavazza A, unpublished observations).

The serological surveys on RHDVs are complicated by the existence of non-pathogenic viruses in the lagoviruses genus, first reported in Italy[20] and then in France[21] and Australia[22] in domestic and wild rabbits. These viruses, named Rabbit Caliciviruses (RCVs), detected starting from serological evidence[16,17,23,24], are

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closely related to RHDV and, in practice, are enteric viruses. The rabbits infected by RCVs are asymptomatic, no macroscopic lesions are observed in the intestine or other organs, and a specific systemic humoral immunity appears within a few days after infection. It should be emphasised that there are significant genetic and antigenic differences between the RCVs discovered in Europe and those found in Australia (RCV A1 - later also identified in Europe), which cause different degrees of interference in RHDV cELISA as well as protection from RHD in rabbits[23,25,26].

RHD is diffused worldwide, mainly where consistent lagomorph populations are present, and its eradication is not feasible in all regions where rabbits are present in the wild. In Africa, the disease was initially observed in the late 1980s; in Tunisia, the first cases of RHD were reported in domestic rabbits in 1992[27]; in Benin, RHD was diagnosed in 1995[28]. In 2020, in Senegal, an RHDV2 outbreak killed many farmed Flemish giants, checkered giants, and Fauve de Bourgogne rabbits[29]. Since 2022, several dispatches from South Africa have reported the presence of RHDV2 in rabbits and hares[15].

Like other Mediterranean countries, Algeria has populations of wild hares and rabbits that cover a large area of the country and constitute a hunting resource. According to Ahmin[30], two hare species have been described: *Lepus capensis*, found from the Northern to the Southern limit of the country, and *Lepus saxatilis*, less abundant, found in the southeast. The wild rabbit *Oryctolagus cuniculus*, known since the 18th century, is present in the North of the country in the forest and coastal regions, as well as in the Tellian Atlas[30,31].

In Algeria, rabbit farming usually occurs in rural areas at a familiar level with small colonies with a mean of 4–5 does[32,33]. However, it is no longer rare to find units with 100 breeding does for commercial purposes, which developed in the 2000s thanks to government support. Most are responsible for meat rabbit production, estimated at 8474 tons[34]. Unfortunately, the progressive intensification and modernisation of rabbit rearing have not been adequately accompanied by farmer training on biosafety measures and health programs[35].

In Algeria, the first RHD case was reported in 2018 in domestic rabbits, followed by other cases in 2020 and 2021, causing economic losses in rabbitries[36]. All these rabbits originated from small farms where no specific control plans for RHD or vaccination were used.

This study presents the results of an investigation aimed at providing the first data on the serological status of farmed rabbits, supporting evidence of the presence, circulation, and distribution of RHDVs in Algeria.

Results

Overview of presence and geographic distribution of RHD-positive Sera in not vaccinated rabbits

We analysed 184 sera collected from 19 farms in eight provinces between October 2020 and May 2022. One hundred three sera (56.0%) were classified as negative for RHD because they were negative in both cELISAs (RHDV and RHDV2) or negative in one ELISA and doubtful in the other. The remaining 81 sera (44.0%) were classified as positive for RHD, having tested positive with a titre of 1/10 in at least one of the cELISA tests. Table 1 shows the percentage of RHD-positive sera for each province. Although in practice, RHD seropositive

Provinces	*n*° sera	% Positive	Districts	*n*° sera	% Positive	Cat.	Sam.
Algiers	11	18	Baba Ali	2	50	B	F
			Oued Smar	4	25	B	F
			Rouiba	5	0	F	M
Bejaia	30	30	Kherrata	26	0	S	M
			Tazmalt	4	100	C	M
Blida	10	30	Soumaa	10	30	B	O
Boumerdes	16	31	Chabet El Ameur	3	33	B	F
			Keddara	3	0	F	M
			Thenia	10	40	B	F
Constantine	2	0	Ain Abid	2	0	F	M
Laghouat	42	100	El Assafia	7	100	S	M
			Ksar El Hirane	14	100	S	M
			Laghouat Center	21	100	S	M
M'Sila	19	5	Boussaada	11	0	S	M
			M'Sila Center	8	13	F	M
Tizi Ouzou	54	44	Makouda 1	20	15	S	M
			Makouda 2	10	20	S	M
			Makouda 3	14	64	S	M
			Makouda 4	10	100	S	M

Table 1. Serological results obtained from unvaccinated rabbits at either the Province or district levels. In the Category (Cat.) column: B = sera collected from breeding rabbits at the farms; C = sera collected from RHD convalescent rabbits at the farms; F = sera collected from fattening rabbits at the farms; S = sera collected from fattening rabbits at the slaughterhouse; The last column (Sam.) indicates the sampling period: O = October 2020, F = February–May 2021 and M = March–May 2022.

unvaccinated rabbits were found in all provinces, their percentage significantly varied, ranging according to the province, from low values (5–20%) in Algiers and M'Sila to intermediate values (30–50%) in Blida, Boumerdes and Tizi Ouzou, to 100% of positive rabbits in Laghouat province.

Table 1 also shows all results obtained on each farm and is representative of individual districts to understand the outcomes of the serosurvey better. In 5 of the 19 farms tested, the sera collected from unvaccinated rabbits (47) were all negative; interestingly, all were sampled from fattening rabbits. These farms were distributed within five provinces, precisely one per province, whereas in the remaining three provinces (Blida, Laghouat, and Tizi Ouzou), all farms tested positive for RHD. Of note, in 8 of the 14 farms found to be seropositive for RHD, sera were collected from fattening rabbits between 75 and 150 days old. Therefore, considering the high RHD titres found in most of these rabbits (see below), these farms, primarily located in Laghouat and Tizi Ouzou provinces, had been undoubtedly affected by RHD outbreaks at most 1–2 months before sampling. Considering that the farm in the Tazmalt district, Bejaia province, was the only one formally declared to be affected by RHD and that this was confirmed by serology obtained on sampled breeding rabbits (see also below), the results obtained by cELISAs established that nine of the 14 farms examined and resulted positive had been affected by RHD shortly before sampling.

Determination of the type of agent responsible for RHD outbreaks in not vaccinated rabbits

Since RHDV and RHDV2 have distinct immunogenic and antigenic profiles, it is frequently possible to compare the titres of a serum obtained in the two cELISAs to determine which lagovirus caused an RHD outbreak. Table 2 shows the percentages of the 81 RHD-positive sera collected from unvaccinated rabbits, assigned to one of five possible categories according to the specific RT value for each examined farm. All four convalescent rabbits, collected from a farm in Tazmalt, were surely or were suspected to be positive due to an RHDV2 infection.

As for RHDV, only one serum was classified as RHDV-infected in Baba Ali district (RHDV cELISA titre 1/160, negative for RHDV2 cELISA), while a second serum from the same farm was negative; both sera have been collected from rabbit breeders older than one year. In addition, we collected 63 sera on the same farm from vaccinated rabbits a few months later to check the effectiveness of vaccination. Still, the results did not indicate any other cases of possible RHDV infection (see below). Two other sera were classified as suspected RHDV infection in farms in the Thenia and Ksar El Hirane districts, but half of the rabbits collected from these farms were also found to have been infected with RHDV2. In all remaining farms, sera were classified as coming from RHDV2-infected or unclassifiable rabbits.

The average RHDV2 titres found in the farms were highly variable, ranging from low to medium titres in breeding rabbits to high titres (≥ 1/1280) in fattening rabbits. Moreover, this last result also correlated, in several cases, with a high percentage of positive sera, reaching 100% in some farms, as all those in the Laghouat province. Unexpectedly, we found significant variability among farms in classifiable and unclassifiable sera proportions. Indeed, while farms such as Makouda 1 and 2 showed a similar proportion to that of the convalescent rabbits in Tazmalt, i.e., all sera were traceable to RHDV2 infection, in Makouda 3, Makouda 4, and Laghouat Center unclassifiable sera were 89, 70 and 62% respectively, and this despite high cELISA titres (see Fig. 1) suggesting

Provinces	Districts	#S	%PS	AT R2	%*R*	%sR	%nc	%sR2	%R2	Cat / age
Convalescent rabbits *n* = 4										
Bejaia	Tazmalt	4	100	1440	0	0	0	50	50	B >365
Unvaccinated healtly rabbits *n* = 77										
Algiers	Baba Ali	2	50	0	100	0	0	0	0	B > 365
	Oued Smar	4	25	40	0	0	0	100	0	B - 120
Blida	Soumaa	10	30	80	0	0	0	0	100	B - 730
Boumerdes	Chabet El Ameur	3	33	20	0	0	100	0	0	B > 365
	Thenia	10	40	380	0	25	25	0	50	B - 180/390
Laghouat	Al Assafeia	7	100	4300	0	0	43	0	57	F - 150
	Ksar El Hirane	14	100	2810	0	7	36	29	29	F - 150
	Laghouat Center	21	100	1600	0	0	62	19	19	F - 150
M'Sila	M'Sila Centre	8	12,5	80	0	0	0	0	100	F - 60
Tizi Ouzou	Makouda 1	20	15	1333	0	0	0	0	100	F - 90
	Makouda 2	10	20	80	0	0	0	50	50	F - 90
	Makouda 3	14	64	2560	0	0	89	0	11	F - 75
	Makouda 4	10	100	4030	0	0	70	10	20	F - 75
Total		137	59		1	3	49	14	32	

Table 2. Rabbits' distribution into distinct categories based on RT value (RHDV2/RHDV titre ratio). #S = number of rabbits sampled; %PS = percentage of RHD positive rabbits; AT R2 = average titre in cELISA RHDV2; %R = percentage of rabbits surely infected by RHDV; %sR= percentage of rabbits suspected of being infected with RHDV; %nc= percentage of rabbits unclassifiable; %sR2= percentage of rabbits suspected of being infected with RHDV2; %R2= percentage of rabbits surely infected by RHDV2; Cat/age= B (Breeding) or F (fattening rabbits) - age in days of rabbits at the time of sampling.

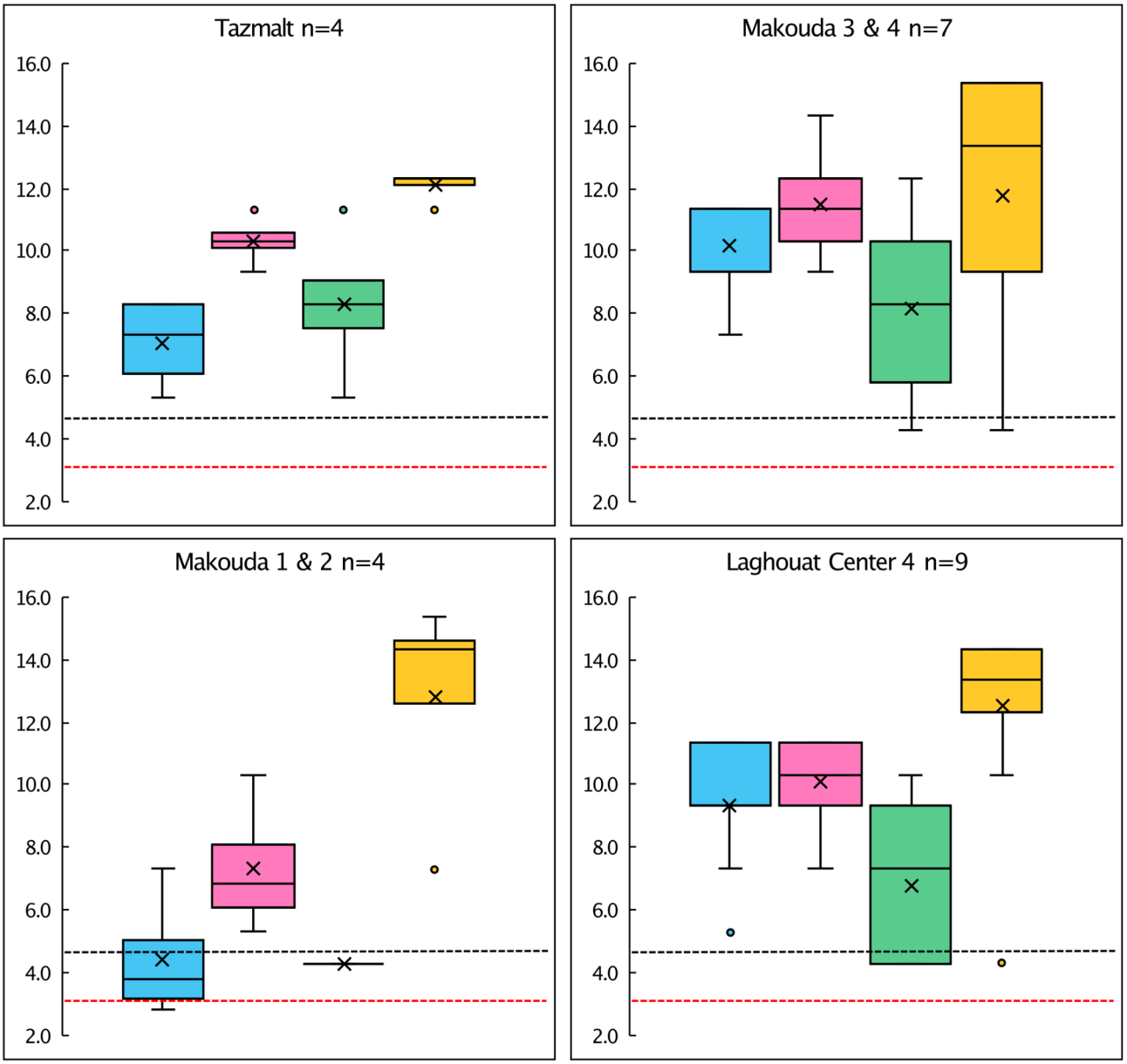

Fig. 1. Box-plot graphs showing cELISA results obtained on farms in relation to RT2 ratio. In the farms in the two left boxes, 100% of the rabbits were correlated with an RHDV2 infection, while in the farms in the two right boxes, over 60% of the rabbits were unclassifiable. Blue = RHDV cELISA; Pink = RHDV2 cELISA; Green IgM-RHDV2 IsoELISA; Yellow = IgA RHDV2 IsoELISA. Red and black dot lines indicate the cut-off value of cELISA and IsoELISA, Log_2 2,8 and Log_2 4,3 respectively. The graphs were obtained using Excel by choosing the median inclusion option. Serum titres are plotted on the y-axis as Log2 of their inverse (i.e. cELISA titre 1/10 equals 3.3). Negative sera were assigned conventional titres of 1/5 and 1/20 for the cELISA and Iso ELISA, respectively.

RHD outbreaks in these farms a few weeks before sampling. However, assigning outbreaks at the farm level was still possible as caused by RHDV2.

Figure 1 shows the cELISA, IgM, and IgA results for farms with different RT2 profiles, according to what is reported in Table 2. Tazmalt convalescent breeding rabbits showed a typical isotype profile of an infection with a high IgA titre (1/5120) and a consistently lower IgM titre (1/320), suggesting an estimated time of infection of 2–3 months before sampling. RT2 values were also typical, ranging from 4 to 32, as evident by the difference in the RHDV/RHDV2 cELISA titre in Fig. 1. Makouda 1 and 2 showed a similar profile to Tazmalt but with all sera negative for IgM and lower titres for both ELISAs. This profile suggests a time of infection (or re-infection) of more than three months before sampling, which is, however, not compatible with the claimed age of the rabbits (90 days). Farms in the last two boxes showed IgM, IgA and cELISA titres indicative of an infection that occurred a few weeks before sampling. Still, they differed in having sera with high RHDV cELISA titres, like those for

RHDV2. Taken together, the data suggested that high cELISA titres for both RHDV2 and RHDV induced by an RHDV2 infection may be related to the early development of humoral immunity in young rabbits.

Serological verification in vaccinated rabbits

The survey was only possible on three farms located in different provinces. The most extensive study was conducted on a farm in the Baba Ali district (Algiers), where we collected 63 sera on several occasions from June to September 2021 on breeding rabbits of around two years old. The last vaccinations for RHDV with Cunipravac RHD (Hipra, Amer, Girona, Spain) and for RHDV2 with Eravac (Hipra, Amer, Girona, Spain) were carried out approximately 3–5 months before the sera sampling. Surprisingly, only seven sera (11%) tested positive for RHD, with rather heterogeneous ELISA results, as shown in Fig. 2.

Of the three sera collected in June, one was only positive in cELISA, one was positive for IgA but not for IgM, and the last was highly positive for both IgM and IgA, indicating a recent RHDV2 infection. Similarly, of the remaining four sera collected in September, three had a low RHDV2 titre, while one was from a rabbit recently infected with RHDV2. Note that the sera collected in July and August, corresponding to 62% of the total, resulted in all negative.

The second farm monitored was in the district of Tigzirt (Tizi Ouzou) (Fig. 3). This farm also used double vaccination of breeding stock (Cunipravac RHD for RHDV and Eravac for RHDV2), with sampling done 5–6 months later. Still, the situation was opposite to the previous one, being positive 11 out of 12 sera (92%). The ELISA titres ranged from 1/40 to 1/640 for both RHDV and RHDV2 and on average, they were somewhat higher for RHDV2, as expected from vaccination with Eravac. While all sera, except one at low titre, were negative for IgM, they showed a certain variability of IgA. In fact, four (36%) of them resulted negative, three (27%) showed low IgA titres (1/320-1/640), and three (27%) medium-high titres (1/2560–1/20480). The last farm monitored was the one in the Chabet El Ameur district (Boumerdes), which was only vaccinated with the Cunipravac RHD one year before sampling. 62% of the sera, collected from 8 breeder rabbits > 1-year-old, resulted RHD-positive but with variable and even high RHDV2 titres, ranging from 1/20 to 1/2560. In addition, three showed high titres of IgM and IhA for RHDV2, indicating that the virus was circulating in the farm shortly before sampling.

Survey on the non-pathogenic lagovirus

To search the farm for the presence of the non-pathogenic Lagovirus RCV-A1, we analysed all the sera that tested negative, doubtful, or 1/10 in RHDV cELISA with the RHDV IgG ELISA, for a total of 97 sera representative of 16 farms. Apart from a few sporadic sera that tested positive at very low titres and, therefore, not indicative of RCV-A1 infection, one positive finding occurred on a farm in Soumaa district (Blida province). All ten rabbits sampled were two years old: three were positive for RHDV2 with low to medium titres, one was negative for all ELISAs, but six were positive only for RHDV IgG ELISA with titres ranging from 1/320 to 1/10,240 (average 1/4000), a clear signature of the presence of RCV-A1.

Discussion

Algeria is an extensive territory covering an area of 2,381,741 square kilometres, divided into 58 territorial authorities called *wilayas* (here named provinces). While family-based rabbit farming is evenly spread throughout the country, commercial farms have been growing since 2000 and are mainly located in the country's

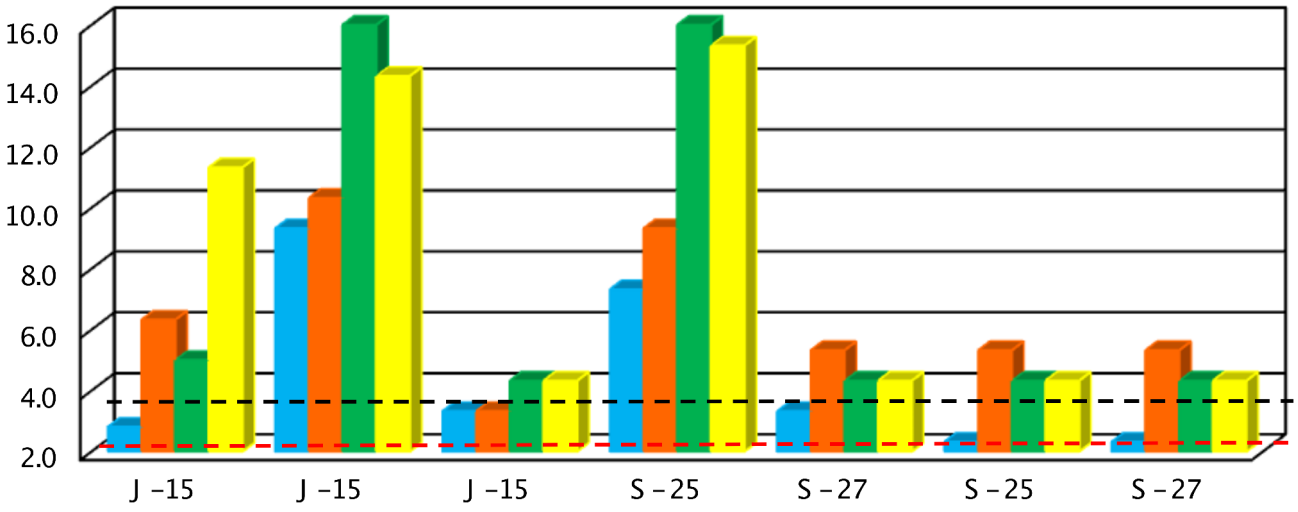

Fig. 2. Complete serological profile of the seven RHD-positive sera in the Baba Ali (Algiers) farm. cELISA RHDV and RHDV2, blue and orange bars, respectively (cut-off red line, Log_2 2,8). IgM and IgA RHDV2, green and yellow bars, respectively (cut-off black line, Log_2 4,3). The numbers in the abscissa indicate the weeks elapsed between the last vaccination and the sampling. The first three sera were collected in June (J) and the last four in September 2021 (S). Serum titres are plotted on the y-axis as Log2 of their inverse (i.e. cELISA titre 1/10 equals 3.3).

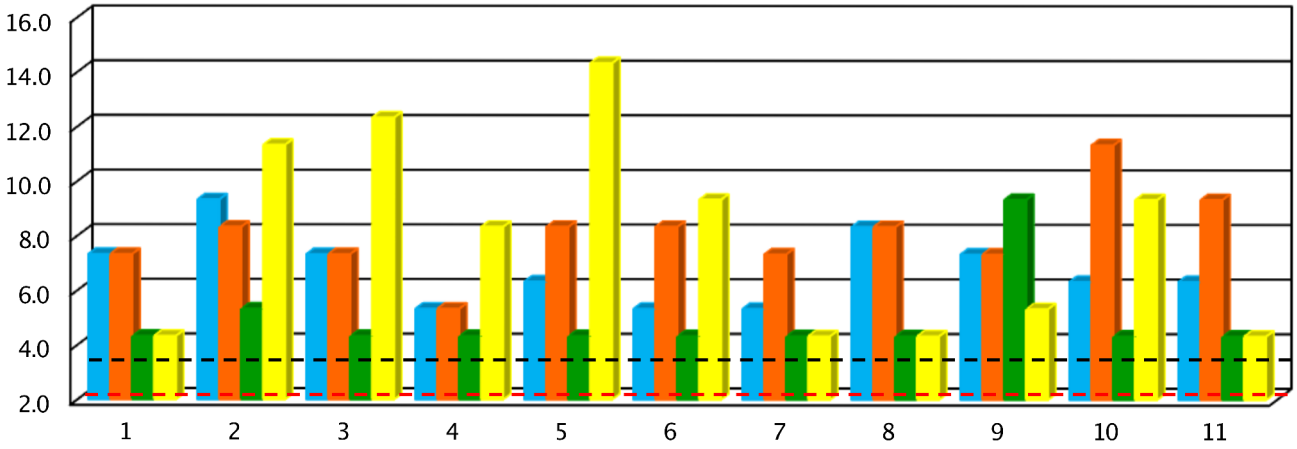

Fig. 3. Complete Serological profile of the 11 RHD-positive sera in the Tigzirt (Tizi Ouzou) farm. cELISA RHDV and RHDV2, blue and orange bars, respectively (cut-off red line, Log_2 2,8). IgM and IgA RHDV2, green and yellow bars, respectively (cut-off black line, Log_2 4,3). Serum titres are plotted on the y-axis as Log2 of their inverse (i.e. cELISA titre 1/10 equals 3.3).

northeastern part. As RHD is undoubtedly one of the leading health problems in rabbit farming, this study aimed to draw a picture of the presence and distribution of the disease in Algeria through a targeted serological survey on rabbit farms, particularly in those areas (about 14% of the Algerian provinces) where rabbit production is more concentrated[35,37–39]. The main difficulty encountered in this study, stating its novelty, was collecting a representative number of blood samples from farms. At the laboratory level, the study used serological methods developed at the WOAH RHD Reference Laboratory, which are widely used worldwide[40–43]. Despite the relatively limited number of samples analysed, the RHD picture obtained for Algeria represents a first and valid starting point for further necessary surveys.

By examining the sera of unvaccinated healthy rabbits, we showed that RHD is widely present in all the provinces included in the survey, albeit with variations in the percentage of affected farms and positive sera between geographical locations. No conclusions can be drawn for the Constantine province since only two sera were collected on a farm. The Algiers, Bejaia, Blida, and Boumerdes provinces should be considered affected by RHD, at least in relation to the sampling time. In fact, of the nine farms involved in the survey, three tested for fattening rabbits were negative, five tested for breeding rabbits were positive with low to medium RHD cELISA titres, and one (Bejaia province) was classified as affected by an RHD outbreak since at least two months, as confirmed by cELISA titres, including the presence of specific IgM in trace (data not shown). The province of M'Sila should be added to the above provinces, as in one farm (Boussaada), all fattening rabbits tested negative, while in a second (M'Sila Center), only one out of eight rabbits fattening rabbits tested positive with a low titre (1/80). Therefore, all these provinces have been affected by RHD but in a different and wide range of time before the sampling. The worst RHD situation was undoubtedly in Tizi Ouzou and Laghouat provinces, where all the not vaccinated fattening rabbits (2–3 months old) from seven farms tested positive for RHD, often in a high percentage of sera, an indication of a significative viral circulation in the population, likely a few weeks before sampling.

As previously reported, the ratio (RT2) between the titres obtained for each serum in the two cELISA often determines whether seroconversion was caused by RHDV or RHDV2, especially in the case of the first infection[6,44]. This is due to the significant antigenic difference between the two viruses, close to being distinct serotypes[3], conjugated with the inherent characteristic of cELISA, in which the interaction between the antigen and serum antibodies occurs in the liquid phase, thus preserving the native conformation of the virus[17]. Accordingly, the overall data confirmed that RHDV2 was the agent causing RHD in Algeria, as reported worldwide. Only in one case, a > 1-year-old not vaccinated breeder from the Baba Ali district (Algiers province), collected in April 2021, the RT2 was indicative of RHDV infection. However, since other sera from rabbit breeders of the same farm collected at the exact moment (one sera) or 2–3 months later (63 sera) resulted mostly negative or in few positive at high titres for RHDV2, it is impossible to support a past RHDV infection in that farm.

The comparison of the RT2 values obtained in each farm requires an interesting additional note. Differently from what is usually found, i.e. most of the sera sampled from RHDV2 convalescent rabbits show an RT2 value ≥ 4, we found some farms where more than 60% of convalescent rabbits had RT2 values between 0.25 and 2 despite cELISA titre in the range 1/2560–1/5120. While the ELISA results confirmed that rabbits from Tazmalt district (Bejaia province) were convalescent, those obtained from Makouda district 1 and 2 farms (Tizi Ouzou province) call into question whether the rabbits sampled were effectively fattening rabbits, at least the few (around 10%) that tested positive considering that their serological profile was compatible with a hypothetical infection that should have occurred before the declared age (90 days). That said, the data obtained in farms with contemporarily high titres for both RHDV2 and RHDV seem to be related to the early stage of antibody production, and this would theoretically be due to two main reasons. The first is the maturation of the antibody response, which, in a short time, selects more affinity and specific antibodies. The second is the switch within

1–2 weeks from the IgM antibodies response, which have a greater capacity to compete in cELISA, given their size, to the IgG-prevalent one. However, the data available in this study were too limited to provide a specific explanation for some of the unexpectedly found RT2 results.

The survey in those few farms applying RHD vaccination for prophylaxis yielded alternative results. Those obtained on the farm in the Baba Ali district (Algiers) were surprising since only 7% of the sera collected from breeder rabbits that received a bivalent vaccination 3–5 months before were seropositive, whereas the expected results should have been over 80–90% positive rabbits with titres 1/40 – 1/640 for both RHDV and RHDV2. Consequently, the only possible conclusion is a vaccination failure on the farm. In addition, the overall data obtained from those few seropositive rabbits raise further doubts: at least two sera, collected in June and September 2021, came from rabbits that were indeed infected with RHDV2 (high IgM and IgA titres, RT2 in favour of RHDV2), showing that such virus was circulating in the farm during those months. Nevertheless, this conclusion strongly disagrees with the 93% of sampled rabbits testing negative during the examined period. In the second farm in the Tigzirt district, almost all vaccinated rabbits tested positive with similar titres for RHDV and RHDV2. However, only one-third of the positivity was likely induced by the sole vaccination (IgM and IgA negative); half of the sampled rabbits resulted in IgM negative/IgA positive, also with high titres. In one serum, IgM with a low titre were found. Considering these results, we can hypothesise that a previous RHDV2 infection may have left a "trace" in the serological data obtained. In the third farm in the Chabet El Ameur (Boumerdes province), where rabbits have been vaccinated one year before only for RHDV, the finding of 40% negative sera and the evidence of infection by RHDV2 occurred about 1–2 months before the sampling reduced the value of the data obtained. This is also considering that one year after vaccination with an aluminium hydroxide-adjuvanted vaccine, the expected results were for a limited number of positive sera with low titres.

To correctly interpret the results of this first serological investigation on RHD in Algeria, it would be necessary also to investigate the possible presence and spread of non-pathogenic lagoviruses (RCVs) in the rabbit populations under study[18]. Besides the clear epidemiological value of this information, its importance is also due to the interference that antibodies induced by the different RCVs can exert on the results obtained in cELISA RHDVs[17]. Actually, only the antibodies induced by RCVs identified in Europe (RCV-Eu) strongly interfere in the RHDV cELISA[23,25], being the antigenic profile between the non-pathogenic and pathogenic viruses very similar[20], whereas, for the same reason, the interference is significantly less in the RHDV2 cELISA. In contrast, the RCVs identified in Australia (RCV-A1), but also circulating in Europe, cause minimal interference in RHDV cELISA and even less in RHDV2 cELISA, being the antigenic profiles of these viruses very different[17,22]. However, whereas the differential serological diagnosis in a farm between RCV-Eu and RHDV needs a multi-approach study, including, for example, simultaneous cELISA testing of does and 2-3-months-old rabbits, the differentiation between RCV-Au and RHDV can be easily done by testing with RHDV IgG ELISA those sera resulted negative or low positive (1/10 – 1/20) in RHDV cELISA[17]. This is due to the property of the IgG ELISA also to detect antibodies produced against the internal common epitopes shared by RCV and RHDV, which, in contrast, are almost undetected by cELISA; accordingly, in the presence of RCV-Au, the sera are usually positive with titres ranging from 1/640 to 1/20,480. We found only one RCV-A1-serologically positive farm in the Soumaa district (Blida province), north-central Algeria. Although previous experiences demonstrated the reliability of results obtained using such an approach, the definitive demonstration of RCV-A1 presence in this farm would require virus identification using already validated diagnostic approaches and genomic tools. As far as possible, more extensive studies on RCVs should be conducted in the different Algerian provinces on more farms, including wild lagomorph populations.

In conclusion, this serological survey highlights two contrasting aspects of the RHD situation in Algeria, even considering that commercial rabbit farming is a recent concept and family breeding is still dominant throughout the country. While RHD due to RHDV2 is widespread in most of the provinces where we looked for it, the effectiveness of surveillance still needs to be improved. In fact, in the provinces with the highest RHD occurrence, young rabbits were slaughtered as 'healthy rabbits' when serological data clearly indicated that they were convalescent and came from farms recently affected by an RHD outbreak.

Moreover, the application of prevention strategies based on the use of vaccines should be extended. Indeed, the results obtained in the few farms surveyed indicate that relevant and careful work is needed to improve the correctness of the vaccination procedure. This could be quickly achieved by following the guidelines in the final report of the Lagmed project[45], of which this survey is also a part and which provides for the preventive health actions that should be implemented to protect farms from RHD better.

Materials and methods

Study area and farm sampling

The serological survey was carried out from October 2020 to May 2022 in 20 rabbit farms from eight provinces (Fig. 4) distributed in north and south of Algeria, as follows: Algiers (n = 3; 36°45′09′′ N/ 3°02′31′′ E), Bejaia (n = 2; 36°45′21′′ N/5°05′04′′ E), Blida (n = 1; 36°28'12'' N/2°49'40'' E), Boumerdes (n = 3; 36°45′59"N/3°28′38′′ E), Constantine (n = 1; 36°21′54′′ N/6°36′53′′ E), Laghouat (n = 3; 33°47′60′′ N/2°51′54′′ E), M'Sila (n = 2; 35°42′21′′ N/4°32′31′′ E) and Tizi Ouzou (n = 5; 36°42′43′′ N/4°02′45′′ E). Most sampled farms (13) were in the northeast area (Fig. 4), mainly in the Tizi Ouzou province, which has the highest density of rabbit farming in Algeria[37,46].

Either local, crossbreeding, exotic, or commercial line (hybrids) rabbits were present in the sampled farms, mostly small-scale farms with 10–60 breeding female rabbits housed in wire cages and fed with commercial pellets. In these farms, planned vaccinations as a method of RHD control were conducted irregularly or were absent. Conversely, in industrial farms with more than 200 does, RHD vaccinations were conducted regularly in breeder rabbits for the RHDV types using Cunipravac RHD and Eravac vaccines.

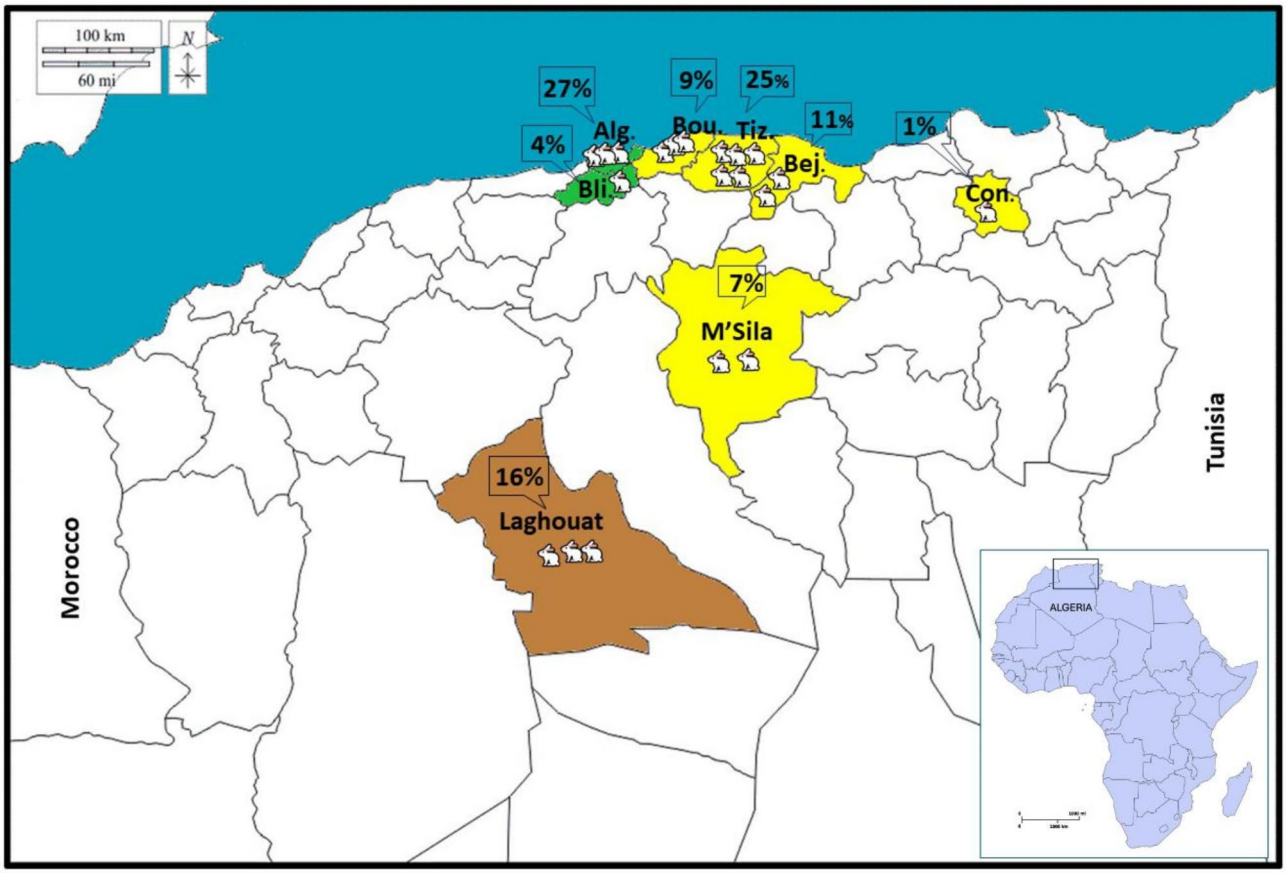

Fig. 4. Map of Algeria showing the geographical distribution of the rabbit farms. The green zones correspond to the provinces in north-central Algeria, the yellow zones to the provinces in north-eastern Algeria, and the brown zone to southern Algeria (Alg. = Algiers, Bej. = Bejaia, Bli. = Blida, Bou. = Boumerdes Con. = Constantine, Tiz. = Tizi Ouzou). The percentage of sera sampled for each province is reported.

Blood collection and rabbit sampling

Two hundred sixty seven blood samples were collected at the farm or the slaughterhouse with the consent of farmers or the abattoir authorities.

The blood was sampled by puncturing the marginal ear vein in rabbits at the farms (tot 134 sera). The age was between 60 and 821 days. Eighty-three rabbits were vaccinated with one or two RHD vaccines. All the rabbits were healthy and came from farms declared free of RHD for over two years, except four rabbits from a farm in Tazmalt, where an outbreak of RHD had occurred a few weeks earlier. At the slaughterhouses, blood was collected from clinically healthy fattening rabbits (tot 133). They were not vaccinated against RHD and aged from 75 to 150 days. Four ml of blood from each rabbit was aseptically collected from the jugular vein using plain vacutainer tubes.

All samples were refrigerated and transported to the laboratory on the same day. The blood samples were centrifuged at 3000 rpm for 10 min, and the sera were stored at – 20 °C until further analysis.

Serological analysis

The serological analysis was performed at the WOAH Reference Laboratory for RHD at IZSLER Brescia in Italy. The first screening was conducted in all sera using two cELISAs specific for RHDV and RHDV2. Selected sera were then analysed with three isotype ELISA tests designed to detect RHDV-specific IgG, RHDV2-specific IgM and RHDV2-specific IgA.

Competitive ELISAs (cELISAs)

The RHDV and RHDV2 cELISA test procedures were performed as previously described[17,19]. Briefly, ELISA 96-well Maxisorp Immuno plates (Nunc, ThermoFisher Scientific Inc, Waltham, MA USA) were coated overnight at 4 °C with a polyclonal RHDV or RHDV2 rabbit serum at a 1:5000 dilution in carbonate buffer (pH 9,6). The plates were then washed with PBS-Tween20 (PBS-T) before serial dilutions (fourfold or sixfold) of rabbit sera, followed immediately by adding the antigen at a fixed limiting concentration. After one hour of incubation and one wash cycle, an HRP-conjugated RHDV-specific MAb or RHDV2-specific MAb was added to the plate to semi-quantify the solid-phase bound antigen. HRP reactivity was developed using OPD at 0.5 mg/ml, and the plate was incubated for 5 min. The plate was then stopped by adding 1 M sulphuric acid. A serum sample was

considered negative if its OD value at the first dilution (1/10) was higher than 85% of the OD value at the same dilution of the negative control serum. Serum was considered doubtful (inconclusive result) if its OD value at the 1/10 dilution was equal to or higher than 75% of the OD value at the same dilution of the negative control serum. A serum sample was considered positive if its OD value at the 1/10 dilution was lower than 75% of the OD value at the same dilution of the negative control serum. The titre of a positive serum sample corresponded to the dilution, causing a 40–60% reduction of the OD value of the negative control serum.

Isotype ELISAs

Isotype ELISAs were performed to detect and titrate IgG for RHDV and IgA and IgM for RHDV2[17,19]. Briefly, to detect RHDV-specific IgG, RHDV-specific MAb is adsorbed to the plate at a concentration of 2 µg/ml using the same procedure described above for the polyclonal serum in the c-ELISA. The virus was added to the plates at a double concentration of the one used in the c-ELISA. After incubation and washing of the plate, sera were added and serially diluted four-fold, starting from 1/40. An MAb anti-rabbit IgG HRP conjugate was used to detect IgG bound to the virus. The HRP reactivity was developed as described for c-ELISA. To detect RHDV2-specific IgM and IgA isotypes, the ELISA reaction steps were reversed to avoid competition with IgG, the predominant isotype in the serum. An MAb anti-rabbit IgM or an MAb anti-rabbit IgA was adsorbed to the wells, and then the sera were diluted and incubated as described above for IgG ELISA. Incubation with the antigen followed, and then a specific HRP-conjugated MAb was used to detect the RHDV2 bound to the plate. A serum sample is considered positive if the OD 492 nm value at the 1/40 dilution is more than 0.2 OD units above the value of the negative serum used as a control. The titre of a positive serum corresponds to the last dilution, which is still positive.

c-ELISAs ratio

Due to a certain degree of cross-reactivity between sera induced by RHDV and RHDV2, to determine which virus had infected the rabbit, we used the ratio of the cELISA RHDV2 titre divided by the RHDV titre (RT2 value)[6,19,44]. If RT2 ≥ 4 or < 4 > 2, the antibodies detected were considered respectively induced or likely induced by RHDV2. If RT2 ≤ 0.25 or > 0.25 < 0.5, the antibodies were considered respectively induced or likely induced by RHDV. Results between 0.5 and 2 were deemed indeterminate and sera unclassifiable.

Data availability

The raw serological data analysed during this study are available upon request from the corresponding author.

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Author contributions

S.M. collected the samples and field data and participated in drafting the first version of the manuscript. L.C. performed the serological test and wrote the manuscript draft. L.S. contributed to the collection of samples and field data. H.L. and H.A. participated in the organization of the field study. L.A. contributed to the discussion and revised the text. C.P. contributed to the serological testing and discussion. All authors critically read the manuscript and approved the final version.

Competing interests
The authors declare no competing interests.

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