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ORIGINAL ARTICLE

Formal estimation of the seropositivity cut-off of the hemagglutination inhibition assay in field diagnosis of influenza D virus in cattle and estimation of the associated true prevalence in Morocco

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Abstract

The influenza D virus (IDV) was discovered less than ten years ago. Increased interest in this virus is due to its nature (RNA virus with high mutation rate), its worldwide circulation in livestock species, its probable role in bovine respiratory disease and its zoonotic potential. Until currently, the establishment of positivity cut-off of the hemagglutination inhibition (HI) assay was not formalized in field conditions for the detection of antibodies directed against IDV in cattle (i.e. the proposed reservoir). In this study, the positivity cut-off of the HI assays was formally established (titre = 10) using a receiver operating characteristic (ROC) curve. This information was used to estimate the sensitivity (68.04 to 73.20%) and the specificity (94.17 to 96.12%) of two different HI assays (HI_1 and HI_2, with two different IDV antigens) relatively to virus micro-neutralization test (VNT) as reference test. Based on the above characteristics, the true prevalence of IDV was then estimated in Morocco using a stochastic approach. Irrespective of the HI assays used, the estimation of the true prevalence was statistically equivalent (between 48.44% and 48.73%). In addition, the Spearman rank correlation between HI titres and VNT titres was statistically good (0.76 and 0.81 for HA_1 and HA_2, respectively). The positive (0.82 and 0.79 for HA_1 and HA_2, respectively) and the negative (0.86 and 0.85 for HA_1 and HA_2, respectively) agreement indices between results of HI assays and VNT were good and similar. This study allowed for a formal establishment of a positivity cut-off in HI assays for the detection of antibodies directed against IDV. This information is of prime importance to estimate the diagnostic sensitivity and specificity of the test relatively to the VNT (i.e. the reference test). Using these characteristics, the true prevalence of IDV should be determined in a country.

KEYWORDS

cattle, epidemiology, hemagglutination inhibition assay, influenza, respiratory, sensitivity, specificity, surveillance, virus

1 | INTRODUCTION

In 2011, a new influenza D virus (IDV) was isolated from pigs with influenza-like clinical signs in Oklahoma, USA (Hause et al., 2014; Hause et al., 2013). Since then, IDV has been detected in a large host range and worldwide, i.e. IDV affected cattle, small ruminants, swine, camelids and equines in America, Europe, Asia and Africa (Dane et al., 2019; Ferguson et al., 2015; Flynn et al., 2018; Murakami et al., 2019; O'Donovan, Donohoe, Ducatez, Meyer, & Ryan, 2019; Oliva, Eichenbaum, et al., 2019; Salem et al., 2017; Silveira et al., 2019; Snoeck et al., 2018; Trombetta et al., 2019).

The zoonotic potential of IDV is still not completely elucidated, but serological and virological studies suggested that the virus might infect human (e.g. Borkenhagen et al., 2018; Hause et al., 2013), especially exposed to cattle (White, Ma, McDaniel, Gray, & Lednicky, 2016). Despite the absence of clinical signs, the virus replicated in the upper respiratory tract of the ferrets (a good model for studying human influenza virus) and a direct contact transmission between ferrets was observed (Hause et al., 2013). In addition, a recent study confirmed that IDV efficiently replicates in an in vitro surrogate model of the respiratory epithelium at temperatures that correspond to the human upper and lower respiratory tract (Holwerda et al., 2019). All these results suggested that human could be susceptible to IDV, but studies are still needed to confirm this hypothesis (Oliva, Mettier, et al., 2019).

Among livestock species, cattle are the most affected host, in which the virus causes moderate respiratory clinical signs alone (e.g. Salem et al., 2019; Ferguson et al., 2016), but may also play a role in the bovine respiratory complex (e.g. Salem et al., 2019; Zhang, Hill, et al., 2019; Zhang, Outlaw, et al., 2019). Consequently, cattle have been proposed as the natural reservoir of IDV (Moreno et al., 2019).

To prevent and control the worldwide spread and inter-species transmission of IDV, options can be proposed such as the development of an efficacious vaccine or the development of antiviral strategies (Asha & Kumar, 2019). Unfortunately, due to the inherent instability and high mutation rate of influenza genomes, most current antivirals are no longer effective at inhibiting influenza virus replication (Kesinger et al., 2018) and the use of antivirus in animals is thus not advised. However, there are some encouraging developments in the field of vaccination. An inactivated influenza D virus vaccine partially protected cattle from respiratory disease caused by homologous challenge (Hause et al., 2017). In another study, a DNA vaccine encoding the consensus hemagglutinin-esterase fusion (HEF) protein of two lineages of IDV (D/OK and D/660) was designed and its efficacy was tested in a guinea pig model. This consensus DNA vaccine elicited high-titre neutralizing antibodies and achieved sterilizing protection against two lineage-representative IDV intranasal infections (Wan et al., 2018; Yu et al., 2017).

The direct diagnosis of the IDV is based mainly on isolation of the virus (e.g. Ferguson et al., 2015) and real-time polymerase chain reaction (PCR) methods (e.g. Faccini et al., 2017). These methods contributed to the understanding of the molecular epidemiology of IDV. The indirect diagnosis of IDV aims at measuring serum antibody to IDV, with mainly virus neutralization (VNT) and hemagglutination inhibition (HI) assays (Moreno et al., 2019). Although there are valuable serologic methods, VNT requires live IDV, well-trained staff and time (Gauger & Vincent, 2020). In addition, a monoclonal antibody-based competitive ELISA test was developed recently and validated for the detection of antibodies against IDV (Moreno et al., 2019). However, in the absence of a commercial ELISA test, the hemagglutination inhibition assay is still often used to estimate the apparent prevalence of the disease. However, the positivity cut-off value of this test had not been formally established.

Further studies are needed to better understand the role of IDV in respiratory infections, especially in cattle, and to evaluate the cost–benefit ratio of mitigation measures (control and prevention) (Oliva, Salem, Meyer, & Ducatez, 2018). To estimate the cost of IDV, the true prevalence of the disease should be assessed. This true prevalence (at population level) can be estimated based on the apparent prevalence using available serological test for which the characteristics must be properly known (sensitivity and specificity) (Rogan & Gladen, 1978).

The first aim of this study was to properly assess the positivity cut-off of HI assays with two distinct antigens relatively to VNT as the gold standard test and using a receiver operating characteristic (ROC) curve, and subsequently, the characteristics of sensitivity and specificity of these assays in the cattle population (i.e. the proposed natural reservoir) and a dataset from Salem et al., 2017. Secondly, the true prevalence was estimated in Morocco based on both the estimation of the characteristics of HI assays and the apparent prevalence.

2 | MATERIALS AND METHODS

2.1 | Data selection

Morocco has 3.2 million head of cattle. A total of 200 cattle sera were sampled between 2012 and 2015, in different geographical areas of Morocco (Table 1, Salem et al., 2017). This sample size was determined using the population size (see above), an expected IDV animal prevalence of 25%, with an accepted error of 6% and a confidence level of 95%, that is, 201 samples (rounded to 200).

2.2 | Laboratory analyses

Hemagglutination inhibition assay and virus micro-neutralization test (VNT) were performed as described previously (Salem et al., 2017).

2.2.1 | Hemagglutination inhibition (HI) assay

The sera were all treated with receptor destroying enzyme (RDE) (Seika) and heme adsorbed on packed horse red blood cells. Four hemagglutination units of D/bovine/Nebraska/9–5/2012 kindly provided by Dr. Ben Hause and subsequently named HI_1 or D/bovine/France/5920/2014 and subsequently named HI_2 and 1%

TABLE 1 Origin[a] of Moroccan bovines sampled during years 2012–2015

Region	Number of sera
Souss-Massa	15
Gharb	22
Zemour–Zair	31
Tadla–Beni Mellal	21
Middle Atlas	25
Saïss	6
Haouz	16
Oriental	5
Chaouia	43
High Rif	12
Oriental High Atlas	4
Total	200

[a]Geographic (bioclimatic) areas with specific husbandry practices

horse red blood cell were used for HI assays. As IDV harbours hemagglutinin-esterase fusion proteins on its surface, which hemagglutinate in presence of red blood cells of chicken, turkey or horse origin, the HI test can be used for IDV antibodies detection.

2.2.2 | Virus micro-neutralization test

The VNT was carried out on swine testis cells (ATCC), using 100 tissue culture infectious doses 50 per well (D/bovine/France/5920/2014) and 5 days' incubation at 37°C and 5% CO2 without TPCK trypsin and an hemagglutination assay as a readout (with 1% red blood cells). We use D/bovine/France/5920/2014 that grows to higher titres on cells than D/bovine/Nebraska/9–5/2012 in our laboratory conditions.

VNT titres tested were <10, 20, 30, 40, 60, 80, 160, 320, 640 and 1,280. VNT titres were considered positive when ≥10. For future statistical analyses, a titre <10 was assimilated as 5 (middle bound value).

Positive IDV reference serum was produced in house by inoculating rabbits subcutaneously with purified D/bovine/Nebraska/9–2/2012. We also used IDV positive French cattle serum (from an experimental infection).

As no negative IDV reference serum was available (specific pathogen free calves do not exist), we used some sera from few cohorts as negative controls, both from the field and uninfected controls from experimental infections (Oliva, Eichenbaum, et al., 2019; Salem et al., 2017, 2019).

2.3 | Statistical analyses

2.3.1 | Receiver operating characteristic (ROC) curve

The receiver operating characteristic (ROC) curve was used to determine the best cut-off of HI_1 and HI_2 related to the VNT as reference. The ROC is a probability curve. The ROC curve was plotted with true positive results (Y-axis) against the false positive results (X-axis). The area under the ROC curve (AUC) is the performance measurement for the classification test at various threshold settings. The higher the AUC, the better the test is able to distinguish between infected and healthy cattle (i.e. measure of the separation of the two subpopulations). In addition, Youden's index 'J' is frequently used in conjunction with the ROC curve analysis, with:

$$\text{Youden's index} = \text{sensitivity} + \text{specificity} - 1 \quad (1)$$

Its value ranges from 0 through 1 (inclusive). A zero value is observed when a diagnostic test gives the same proportion of positive results for groups with and without the disease. A value of 1 indicates that there are no false positives or false negatives, i.e. the test is perfect. In a ROC curve, the calculation of Youden's index in all points allows to determine the best cut-off of the test (i.e. optimal Youden's index).

2.3.2 | Estimation of the relative sensitivity, specificity of the hemagglutination inhibition assays and the true prevalence

The sensitivity and the specificity of the HI assays were estimated based on the cut-off established using the ROC curve and relatively on the results of the VNT as reference test. The 95% confidence interval of each parameter was estimated based on exact binomial distribution, using STATA/SE 14.2 (StataCorp.).

The animal true prevalence (TP) of IDV was estimated from the apparent prevalence (AP) calculated in this study using HI assays (i.e. the seroprevalence) and the individual diagnostic specificity (Sp) and sensitivity (Se) based on the ROC curve, using the Rogan and Gladen formula (1978):

$$TP = (AP + Sp - 1) / (Se + Sp - 1) \quad (2)$$

For the AP, Sp and Se values of different HI assays, a uniform variable was used, taking into account the extreme values of the 95% confidence interval, and a stochastic modelling (1,000 Monte Carlo simulations) was performed using @Risk 7.5.2 software (Palisade Corporation, Ithaca, New York, USA) to estimate the TP with a 95% CI using Equation (2).

2.3.3 | Agreement between hemagglutination inhibition assays and virus micro-neutralization test

The correlation between the titres of HI assays and the VNT was assessed using the Spearman rank correlation, a non-parametric test (Petrie & Watson, 2013).

The two tests (positive and negative results using the cut-off selected) were compared using concordance analysis to assess their

agreement with the results. The level of agreement was expressed in terms of Cohen kappa coefficient (κ) (Petrie & Watson, 2013) and of indices of positive (P_{pos}) and negative agreement (P_{ne}) (Cicchetti & Feinstein, 1990), the observed agreement proportion for positive and negative test results, respectively. Confidence intervals were calculated using an exact binomial distribution.

The P_{pos} and P_{ne} were used to resolving the paradoxes of Cohen kappa coefficient (conditions when there is a high agreement but low kappa) (Cicchetti & Feinstein, 1990).

Using a 'two-by-two' contingency table (Table 3), the two indices of positive agreement (P_{pos}) and negative agreement (P_{neg}) were, respectively,

$$P_{pos} = 2a/(2a+b+c) \quad (3)$$

and

$$P_{neg} = 2d/(2d+b+c) \quad (4)$$

where P_{pos} and P_{neg} were the indices of positive and negative agreement, respectively (parameters *a*, *b*, *c* and *d* are detailed in Table 3).

In addition, the comparison of titres obtained in HI assays and VNT was assessed with a non-parametric two-sample Wilcoxon rank-sum test and using a Bonferroni correction for multiple comparisons (alpha was fixed as 0.017, i.e. 0.05 divided by 3 comparisons).

3 | RESULTS

3.1 | Titres of hemagglutination inhibition assays and virus micro-neutralization test

The titres obtained for the three tests (HI_1, HI_2 and VNT as reference) are depicted in Table 2 and Appendix S1. High titre of antibodies was observed for all the three tests. While titres of HI_1 and HI_2 are similar (two-sample Wilcoxon rank-sum test; *p*-value = .51), titres of VNT are significantly higher than titres of HI_1 and HI_2 (two-sample Wilcoxon rank-sum test; *p*-value ≤ .0001). Besides, according to Table 2, VNT was more sensitive than HI at higher titres, but less sensitive than HI at lower titres.

TABLE 3 Comparison of HI_1 and VNT results for influenza D in 200 cattle samples from Morocco

	HI_1		
	Positive	**Negative**	**Total**
VNT			
Positive	71*(a)*	26*(b)*	97
Negative	6*(c)*	97*(d)*	103
Total	77	123	200

Note: (a), (b), (c) and *(d)* are parameters used in Equations (3) and Equation (4).

TABLE 2 Number of sera obtained in function of the assays and the titres obtained

Titre	VNT	HI_1	HI_2
<10	103	123	130
10	8	30	29
20	13	15	15
30	1		
40	21	22	9
60	1		
80	16	6	8
160	14	2	6
320	15	1	2
640	4	1	1
1,280	4		

Note: HI_1, hemagglutination inhibition assay using D/bovine/Nebraska/9-5/2012; VNT; HI_2, hemagglutination inhibition assay using D/bovine/France/5920/2014; VNT virus micro-neutralization test using D/bovine/France/5920/2014.

3.2 | Receiver operating characteristic (ROC) curve

The best cut-off for HI_1 or HI_2 was estimated based on the VNT as reference test and using a ROC curve (Figure 1). For the two ROC curves, the best positivity cut-off was the same, with a titre = 10. At this point estimate, the optimal Youden's indexes were observed and reached 0.67 and 0.64 for HI_1 and HI_2, respectively.

The area under the ROC curve (AUC) for HI_1 and HA_2 was, respectively, 0.84 (standard error = 0.026) and 0.83 (standard error = 0.025). The difference between these areas was not significant ($\chi^2 = 0.34$; *p*-value = .56).

3.3 | Estimation of the sensibility and specificity of hemagglutination inhibition assays relative to the micro-neutralization as reference test

Based on the cut-off determined by the ROC curve for HI_1 and HI_2, the contingency tables were established for HI_1 and HI_2, respectively (Tables 3 and 4).

The sensitivity and the specificity of the HI_1 related to the VNT were 73.20% (95% CI: 63.24–81.68) and 94.17 (95% CI: 87.75–97.83), respectively. Youden's index for HI_1 was 0.67.

The sensitivity and the specificity of the HI_2 related to the VNT were 68.04% (95% CI: 57.80–77.15) and 96.12 (95% CI: 90.35–98.93), respectively. Youden's index for HI_1 was 0.64.

3.4 | Estimation of the apparent and true prevalence

The apparent prevalence of IDV was estimated with HI_1 and HI_2, as, respectively, 38.50% (95% CI: 31.72–45.62) and 35.00% (95% CI: 28.41–42.05). There was no statistical difference in the

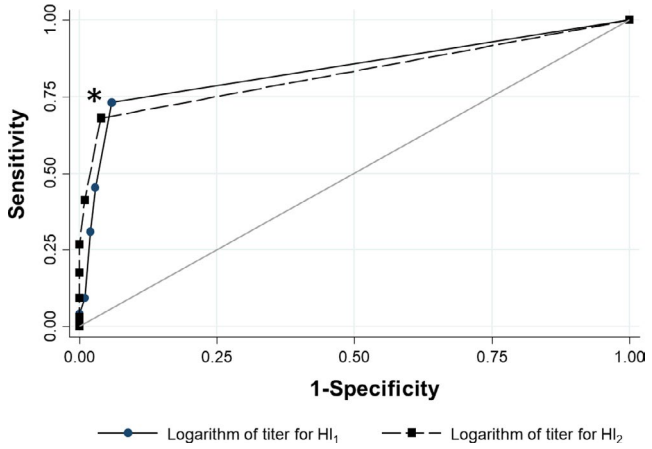

FIGURE 1 Receiver operating characteristic (ROC) curve based on hemagglutination inhibition assays and virus micro-neutralization test as reference. HI_1, hemagglutination inhibition assay using D/bovine/Nebraska/9-5/2012; VNT; HI_2, hemagglutination inhibition assay using D/bovine/France/5920/2014; the reference test was the virus micro-neutralization test using D/bovine/France/5920/2014. The asterisk indicates the best cut-off of the two HI assays (titre = 10)

estimation of the apparent prevalence using HI assays ($\chi^2_{(1df;0.05)} = 0.53$; p-value = .47).

Based on the sensitivity and the specificity of HI_1 and HI_2 and their above estimated apparent prevalence, the true prevalence was estimated using the Rogan and Gladen formula (1978): 48.73% (95% CI: 34.06–64.40) with HI_1 and 48.44% (95% CI: 33.69–65.14) with HI_2 (Figure 2). These two estimations were concordant ($\chi^2_{(1df;0.05)} = 0.1$ (p-value = .92).

3.5 | Level of agreement between tests

The Spearman rank correlation between the logarithm of the titre of HI_1 and the logarithm of the titre of VNT was 0.76 (p-value < .0001). The Spearman rank correlation between the logarithm of the titre of HI_2 and the logarithm of the titre of VNT was 0.81 (p-value < .0001) (Figure 3). These correlations indicated a good relation between the titres of different tests used.

According to the cut-off of HI_1 and HI_2 determined previously (i.e. titre = 10), the level of agreement with the results of the VNT was calculated. The two-by-two contingency tables for two different HI assays are depicted in Tables 3 and 4.

These HI assays were in good agreement with VNT (using the scale proposed by Petrie & Watson, 2013) as the Cohen kappa coefficient (κ) was of 0.68 (95% CI: 0.54–0.81) (p-value < .05) and 0.65 (95% CI: 0.51–0.78) (p-value < .05) for HI_1 and HI_2, respectively.

The positive agreement index (Cicchetti & Feinstein, 1990) between HI assays and VNT was 0.82 (95% CI: 0.75–0.78) and 0.79 (95% CI: 0.72–0.85) for HI_1 and HI_2, respectively.

The negative agreement index (Cicchetti & Feinstein, 1990) between HI assays and VNT was 0.86 (95% CI: 0.81–0.90) and 0.85 (95% CI: 0.80–0.89) for HI_1 and HI_2, respectively.

The positive and negative agreement indices were not significantly different.

TABLE 4 Comparison of HI_2 and VNT results for influenza D in 200 cattle samples from Morocco

	HI_2		
	Positive	**Negative**	**Total**
VNT			
Positive	66	31	97
Negative	4	99	103
Total	70	130	200

4 | DISCUSSION

The interest of IDV resides in the fact that the virus was discovered recently (Hause et al., 2014; Hause et al., 2013), harbours a RNA genome with a high rate of mutations (Kesinger et al., 2018), circulates worldwide in livestock, may play a role in the bovine respiratory complex (Salem et al., 2019; Zhang, Hill, et al., 2019; Zhang, Outlaw, et al., 2019) and has a zoonotic potential (e.g. Borkenhagen et al., 2018; Hause et al., 2013; Holwerda et al., 2019).

In this study, the positivity cut-off of the HI assays was formally established (titre = 10) for cattle in field conditions using a ROC curve. This information was used to estimate the sensitivity (between 68.04% and 73.20%) and the specificity (between 94.17% and 96.12%) of HI assays relatively to the VNT as reference test. Based on the above characteristics, the true prevalence of the IDV was then estimated in Morocco using a stochastic approach. Irrespective of the antigen used in HI assays, the estimation of the true prevalence was statistically equivalent (between 48.44% and 48.73%). In addition, the Spearman rank correlation between HI and VNT titres was statistically good (0.76 and 0.81 for HA_1 and HA_2, respectively). The positive (0.82 and 0.79 for HA_1 and HA_2, respectively) and the negative (0.86 and 0.85 for HA_1 and HA_2, respectively) agreement indices between HI assays and VNT were good and similar. In addition, the VNT was also less sensitive than HI at lower titres. In humans, for influenza A, a good correlation between HI and VNT titres and the lesser sensitivity than HI at lower titres were also previously observed (Truelove et al., 2016). Like for influenza A, the reason is unknown and needs more investigation (Truelove et al., 2016). Indeed, as for influenza A (Truelove et al., 2016), the use of HI assay for detection of antibodies against IDV is supported over the more resource intensive VNT (i.e. requires cell culture, more time and labour, and technical skill to conduct the assay compared to other serological methods) (Gauger & Vincent, 2020).

Despite the genetic distance between the hemagglutinin-esterase fusion (HEF) genes of the two strains of IDV (Chiapponi et al., 2019), no statistical difference was observed in the

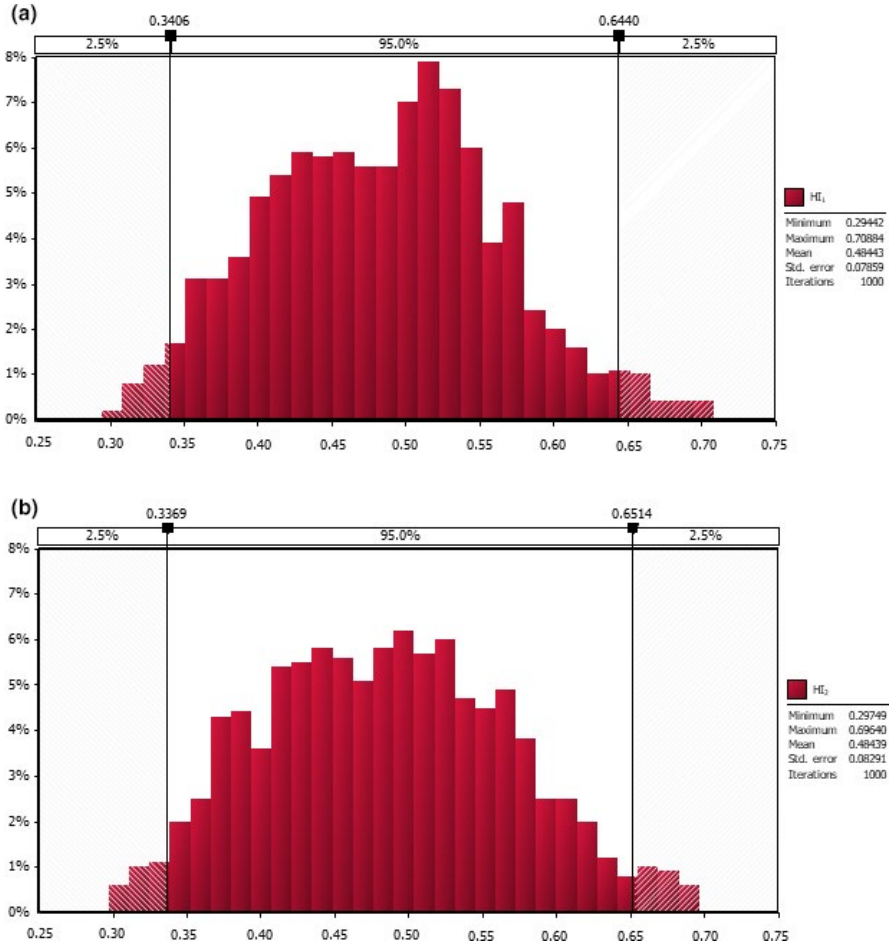

FIGURE 2 Stochastic estimation of the true prevalence based on hemagglutination inhibition assays ([a] = HI_1; [b] = HI_2) (1,000 iterations)

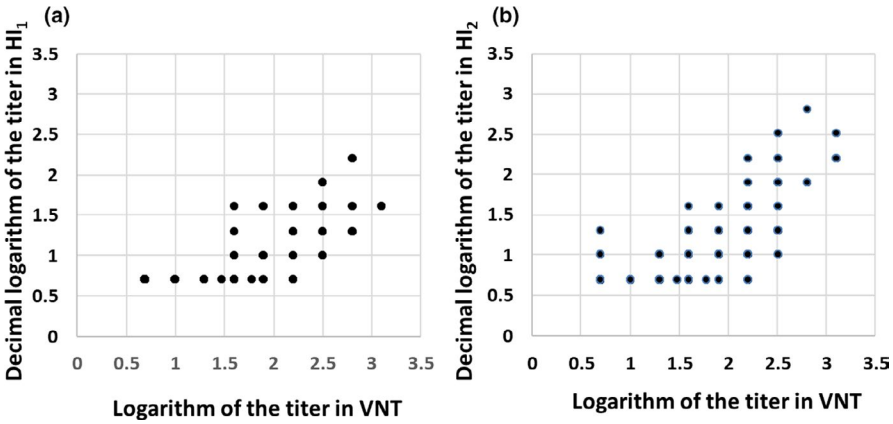

FIGURE 3 Correlation between hemagglutination inhibition assays ([a] = HI_1; [b] = HI_2) and the virus micro-neutralization for the detection of antibodies directed against influenza D virus

characteristics of the two HI assays. Indeed, the same formal cut-off can be proposed for both tests. The antigenic differences between the two viruses therefore do not alter the degree of detection of anti-IDV antibodies in this cohort of Moroccan cattle sera. However, similar studies regarding other species are needed.

Until currently, the establishment of cut-off of HI assays was not formalized for the detection of antibodies directed to IDV. Previous studies have used titres of 10–40 as thresholds for IDV positive antibody titres (Luo et al., 2017; Salem et al., 2017). This establishment is of prime importance to assess the diagnostic sensitivity and specificity of HI assays, to compare seroprevalence studies (and thus geographical areas) and to calculate the true prevalence of IDV. In addition, the recent development of monoclonal-based competitive ELISA used HI as reference test (Moreno et al., 2019) and emphasizes the importance of the establishment of a formal cut-off for any HI assay.

Among the mitigation measure against IDV, the vaccination is a credible option (e.g. Wan et al., 2018), especially in its main host, i.e. cattle (Dane et al., 2019; Salem et al., 2019). The choice of any strategy is based on the estimation of the cost–benefit ratio (e.g. Renault et al., 2019; Souley Kouato et al., 2018). Indeed, to be able

to quantify the cost (in monetary terms), one needs the estimation of the true prevalence of IDV. For the development of future ELISA tests (i.e. screening tools for the detection of antibodies against IDV), a validation with at least an HI assay (e.g. Moreno et al., 2019) followed by inference of VNT using the methodology developed in this study or by the direct use of VNT as reference test is recommended.

One limitation of this study is the absence of complete sampling design, which needs a good knowledge of the sampling frame. However, sera of this study were sampled in numerous regions of Morocco to give a good representability of the cattle population.

The present study and methodology should be extended to samples from other continents (and/or other circulating strains of IDV) and other species in the purpose to consolidate the formal positivity cut-off of HI assays proposed here.

5 | CONCLUSION

This study allowed to formally establishing the positivity cut-off of the HI assays for the detection of antibodies directed against IDV. This information is of prime importance to estimate the diagnostic sensitivity and specificity of the test relatively to the VNT, which is the reference test. Using these characteristics, the true prevalence of IDV should be determined in a country.

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CONFLICT OF INTERESTS

The authors declare that they have no competing interests.

ETHICAL APPROVAL

Ethical statement is not applicable to this study as the raw data were gathered through part of the study form Salem et al. (2017) without any additional animal experimentation.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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