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A retrospective evaluation of pooled serum ELISA testing in the frame of the French eradication program for infectious bovine rhinotracheitis

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ABSTRACT

Pooled serum testing using whole-virus indirect ELISA has been recently recognized as an official method for surveillance of bovine herpesvirus 1 (BoHV1) in cattle herds in Europe. In this study, a retrospective analysis of data from the French BoHV1 surveillance campaign 2018–2019, including 7434 BoHV1-free certified herds and 157 infected herds, was performed in order to evaluate the diagnostic specificity and sensitivity of two pooled serum indirect ELISAs (from IDEXX and IDVet), in comparison with individual testing by blocking ELISAs targeting the gB and gE proteins. Pooled serum testing showed a relative specificity higher than 97.5% and a detection rate of 100% since all gB+/gE+ samples were found in positive pools. At the herd level, no more than one false positive pool was observed in most of BoHV1-free certified herds, leading to a herd relative specificity of 85.1% and 86.0% for the IDEXX and IDVet pooled serum ELISAs, respectively. Among infected herds tested by pool sizes up to 10 sera ($n = 122$), 46% of herds were detected through pools of size 10 containing a single positive sample, 23% through pools of size 10 containing at least two positive samples, and 31% through pools of smaller sizes. A complementary study based on manually constituted pools revealed that at least one positive sample in 100% and 93.4% of herds could be detected individually by pools of size 10 with the IDEXX and IDVet ELISAs, respectively. However, pooled serum ELISAs were influenced by the level of individual reactivity, since pools composed of either one weak-positive sample or one gB+/gE- sample could yield negative results. Altogether, these results provided the first evidence that pooled serum testing (pool size up to 10) is a suitable strategy for surveillance of BoHV1-free cattle farms.

1. Introduction

Infectious bovine rhinotracheitis (IBR) is an important disease of livestock industry caused by bovine herpesvirus 1 (BoHV1), a virus of the genus *Varicellovirus* in the subfamily *Alphaherpesvirus*. Acute infections generally cause upper respiratory tract disease and conjunctivitis (Straub, 1991). The virus can also infect the genital tract, leading to infectious pustular vulvovaginitis (IPV) and balanoposthitis (IPB) in cows and bulls, respectively (Pastoret et al., 1982). BoHV1 infections are often subclinical and mortality is low. After recovery, animals become lifelong latent carriers of the virus. Reactivation may be triggered by natural and artificial stressful events (parturition, diet, dramatic weather conditions, transport and animal mixing, immunosuppression or treatments with corticosteroids), leading to shedding and

transmission of the virus via the respiratory, ocular or genital secretions as well as the semen from infected bulls (Thiry et al., 1985, 1987; Turin et al., 1999; Winkler et al., 2000; van Drunen Littel-van den Hurk, 2006; Jones and Chowdhury, 2010).

BoHV1 infection can induce direct production losses through reduced milk yield, weight loss, abortions, infertility, and higher susceptibility to secondary bacterial infections (Hage et al., 1998; Nandi et al., 2009). Several member states of the European Union (EU) have either successfully eradicated IBR (Austria, Denmark, Germany, Finland, Sweden) or implemented an EU-approved compulsory program at the national or regional scales (Belgium, Czech republic, France, Italy) (Iscaro et al., 2021). These programs rely on regular serological testing combined with vaccination and/or removal of positive animals (Raaperi et al., 2014). Three distinct commercial ELISA tests are available for

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BoHV1 diagnosis, including a whole-virus indirect ELISA and two blocking ELISAs based on the detection of specific antibodies against the viral glycoprotein B or E (gB and gE ELISAs, respectively). Whole-virus indirect ELISA is suitable for the detection of BoHV1 antibodies in milk samples (Kramps et al., 2004; Tignon et al., 2017), and is currently used for surveillance of dairy farms by bulk milk testing. The gB ELISA shows the best performance on serum, with a diagnostic specificity and sensitivity higher than 99% (de Wit et al., 1998; Kramps et al., 2004; Valas et al., 2019), and is commonly used for testing individual blood samples from dairy farms yielding positive results by bulk milk testing and for surveillance of beef farms. The gE ELISA was developed to differentiate infected animals from animals vaccinated with gE-deleted strains (Strube et al., 1996; Van Oirschot et al., 1997). This test is intrinsically less sensitive than the indirect and gB ELISAs on serum and milk samples, showing a relative sensitivity of 93–98% (Van Oirschot et al., 1997; Wellenberg et al., 1998a; de Wit et al., 1998; Kramps et al., 2004; Tignon et al., 2017). However, the gE ELISA exhibits a diagnostic specificity of 100% (de Wit et al., 1998; Wellenberg et al., 1998a; Kramps et al., 2004; Tignon et al., 2017), and can be useful as confirmatory test to identify nonspecific reactions by indirect and gB ELISAs induced by antigenically BoHV1-related alphaherpesviruses (Böttcher et al., 2012; Valas et al., 2019; Petrini et al., 2020).

According to former EU regulations, pooled sample testing could be performed only on milk samples for the detection of BoHV1 antibodies. On April 21st, 2021, a new EU regulation and its delegated acts (directive 2016/429) went into force, also known as the Animal Health Law (AHL). This new legal framework approves the use of pooled sample testing on serum. However, while bulk milk testing can be used for the acquisition and maintenance of the IBR-free status, pooled serum testing is restricted to surveillance purposes. Bulk milk testing is a fast, non-invasive and cost-effective method, but exhibits a lower sensitivity than methods based on individual testing. Indeed, the herd level relative sensitivity of blocking ELISAs applied to bulk tank milk (BTM) ranged from 76.5% to 85.7% (Wellenberg et al., 1998b; Nylin et al., 2000; Raaperi et al., 2010). In addition, the detection of herds with a low prevalence is uncertain since BTM may represent only a fraction of cows, excluding dry cows, sick cows or cows in the colostrum period. These diagnostic drawbacks have been compensated by limiting the number of cows in a pool (pools of up to 50 and 100 milk samples for the acquisition and monitoring of the BoHV1-free status, respectively) and/or by increasing the frequency of sampling (every 3 months). Sampling based on serum pools remains a cost-effective approach that allows testing of all animals in beef and dairy herds. Moreover, the number of individual samples in serum pools is lower than in BTM, virtually limiting the influence of the dilution effect on pool sensitivity (Nylin et al., 2000). However, partitioning of sera into pools requires that the test is sensitive enough to detect a single positive sample in the pool. To our knowledge, the reliability of pooled serum testing for the detection of antibody response towards BoHV1 has not been documented yet.

While bulk milk testing has been used in most of European countries with an EU-approved eradication program of BoHV1, pooled serum testing has been carried out only in France. Briefly, testing of pools up to 10 sera by indirect ELISA is performed annually on animals older than 24 months from beef farms and on lactating cows from farms yielding a positive result in BTM. All individual samples from positive pools are retested by gB ELISA. Since bovine herpesvirus 2 (BoHV2) is prevalent in France and can induce nonspecific reactions in indirect and gB ELISAs (Böttcher et al., 2012; Valas et al., 2019; Petrini et al., 2020), gE ELISA is used to further verify non-negative results (doubtful and positive reactions) in gB ELISA.

In this study, the diagnostic performance of whole-virus indirect ELISAs on serum pools was estimated retrospectively by using data from the surveillance campaign 2018–2019 for IBR in 12 French departments. Positive reactions by pooled serum ELISAs were compared with results of gB/gE individual testing in BoHV1-free certified herds and herds with new BoHV1 outbreak. In addition, individual testing was performed on

sera composing negative pools from a panel of infected herds in order to detect any diagnostic failure. Finally, the reliability of pooled serum ELISAs was further evaluated through pools experimentally composed of field singleton reactors and weak-positive samples.

2. Materials and methods

2.1. Data sources and collection of field samples

Serological data of BoHV1 surveillance performed between October 2018 and July 2019 in 12 French departments were investigated in the current study. Four departments provided data only from infected herds, one department provided data only from BoHV1-free certified herds, and seven departments provided data from both types of herds. In each department, identification and type (beef, dairy or mixed) of cattle herds eligible for the present study were supplied by the animal health protection farmers' organizations (GDS) in charge of the implementation of the IBR compulsory eradication program. Animal-level information for each selected herds (animal identification, date of sampling, sizes of pools in pooled serum testing, kits used for routine diagnosis and serological data) was extracted from databases of official laboratories (OL) and compiled at the National Reference Laboratory (NRL). In total, serological data of 57,734 serum pools from 7434 BoHV1-free certified herds and 1866 serum pools from 157 BoHV1-infected herds were used for evaluation of the performance of pooled serum ELISA kits. The maximum size of pools did not exceed 10 sera. For a subset of 75 infected herds, all individual sera submitted to BoHV1 diagnosis and stored at the OL were transferred to the NRL for further analysis in order to evaluate whether pooled sample testing could yield false negative results.

2.2. Selection of cattle herds

Serological results of IBR surveillance in beef cattle farms were used for the evaluation of the diagnostic specificity of ELISA kits on pooled sera. Herds were selected according to the following criteria. They tested seronegative in at least two consecutive controls performed at a minimum 3-month interval on all animals older than 12 months before the beginning of the campaign 2018–2019, did not contain IBR-vaccinated animals, and any individual blood sample detected positive by gB ELISA during the campaign 2018–2019 was not confirmed by gE ELISA. Results of IBR monitoring in BoHV1-free dairy cattle farms were excluded for two reasons. First, these herds are monitored by serological bulk milk testing, and blood samples are not available in case of negative results in BTM. Second, positive results in BTM would have inherently introduced a bias in the evaluation of the specificity of blood testing.

Evaluation of the diagnostic sensitivity of ELISA kits on pooled sera was performed on the basis of serological results from dairy, beef or mixed (dairy/beef) cattle farms which experienced a least one gE-positive result on individual blood sample during the campaign 2018–2019 (case definition of an infected herd). Herds with three distinct IBR statuses at the beginning of the campaign 2018–2019 were investigated in this study, including 34 BoHV1-free certified herds without IBR-vaccinated animals, 7 herds without IBR-vaccinated animals and showing a favorable serological profile (one negative serological blood testing of all cattle older than 12 months for beef farms, or two consecutive negative controls by bulk milk testing at a minimum 4-month interval for dairy farms), and 116 vaccinated herds composed of a mix of seronegative animals and vaccinated/infected animals. Herds with less than 10 animals eligible for IBR surveillance were excluded.

2.3. ELISA kits

Detection of BoHV1 antibodies at the OL was performed by using commercially available and registered ELISA kits, including two pooled serum indirect ELISAs (IDEXX IBR Pool [IDEXX] and ID Screen IBR Mixte Indirect [IDVet]), three gB blocking ELISAs (IDEXX IBR gB X3

[IDEXX], ID Screen IBR gB competition [IDVet] and BioLISA kit IBR gB Ab [Biosellal]), and three gE blocking ELISAs (IDEXX IBR gE [IDEXX], ID Screen IBR gE competition [IDVet] and BioLISA kit IBR gE Ab [Biosellal]). All kits were submitted to a preclicensing control and batch releasing test before their distribution on the French market. Each OL used one kit of each category (indirect/gB/gE) during the whole campaign, except one OL which switched from the IDVet to the Biosellal gE kits. Pooled serum ELISAs had one protocol (short incubation) and one cut-off value to discriminate between positive and negative results (50% and 45% for IDEXX and IDVet kits, respectively). All gB ELISA kits had two cut-off values delimiting a doubtful range (65%–75% for IDEXX, 25%–35% for IDVet and 60%–70% for BioLISA). The IDVet and BioLISA gE kits contained one cut-off value (60% and 35%, respectively), while the IDEXX gE ELISA had a doubtful range (60%–70%). Doubtful results by blocking ELISAs were considered as positive, according to the analytical rules laid down by the French eradication program. At the OL level, individual sera from positive pools were retested by gB blocking ELISA. Those with a gB non-negative result (doubtful or positive reaction) were then submitted to a gE blocking ELISA. Positive results by gE ELISA were classified as BoHV1-positive reactions. Confirmatory tests were carried out at the NRL on individual sera from negative pools using the IDVet gB and IDEXX gE blocking ELISAs. Like in OL, sera were first tested by gB ELISA and those yielding a gB non-negative result were analyzed by gE ELISA. All assays carried out at the OL and NRL were performed according to manufacturer's guidelines and using prescribed cut-off values.

2.4. Evaluation of the performance of pooled serum ELISAs

The blocking ELISAs performed on individual sera were used as reference tests for calculating the performance of pooled serum ELISAs. The relative diagnostic specificity was estimated using data from BoHV1-free certified herds and was defined as the proportion of pools containing only gB-negative individual samples that yielded a negative result. Negative pools were stated as true negative, and positive pools without gB-positive individual sera were classified as false positive. Positive pools containing gB-positive sera were considered as true positive and excluded for the estimation of the diagnostic specificity. The herd specificity was defined as the proportion of BoHV1-free certified herds with no false positive pools. The relative diagnostic sensitivity of pooled sample testing was estimated using data from the subset of 75 infected herds for which individual sera composing both positive and negative pools were tested by blocking ELISA. The sensitivity was defined as the proportion of pools containing at least one gB+/gE+ individual sample that yielded a positive result. Since gB ELISA could yield nonspecific reactions due to cross-reactivity with other alphaherpesviruses, only samples tested positive by gB and gE ELISAs (gB+/gE+ sera) were classified as true positive. Consequently, positive pools containing gB+/gE+ sera were classified as true positive, negative pools containing gB+/gE+ sera were classified as false negative, while pools only containing gB-positive sera were excluded for the estimation of the diagnostic sensitivity.

To evaluate the ability of pooled serum ELISAs to correctly identify pools of size 10 containing a single gB+/gE+ sample, 100 gB+/gE+ samples from 61 infected herds were tested individually in pools manually reconstituted at the NRL. Each positive sample was diluted at 1:10 in a mix of nine individual ELISA negative samples and then tested by the IDEXX and IDVet pooled serum ELISAs. Among the 100 gB+/gE+ samples investigated, 30 samples yielded reactions within the dynamic range of the gE ELISA (near the cut-off value) and were classified as gE-weak positive samples. Those showing a reaction above the dynamic range of the kit were classified as strong positive samples.

2.5. Statistical analysis

Statistical analysis was performed using the R software version 4.1.2

(R Core Team, 2021). The proportions of false-positive results between any two sizes of pool for each indirect ELISA were compared using the mid p-value method to check for statistically significant differences (Agestri and Gottard, 2007). Data from one OL which only used pools of size 8 were excluded from the analysis. The proportions of gB+/gE-samples between infected and BoHV1-free certified herds were compared using the same statistical method. The confidence intervals for the relative diagnostic sensitivity and specificity of pooled serum ELISAs were based on the exact binomial method. The agreement between pooled serum ELISAs and individual gB/gE blocking ELISAs was calculated as the proportion of concordant interpretations (positive and negative results). The level of agreement between methods was evaluated using the Cohen's kappa coefficient (κ) (Cohen, 1960), and should be considered as almost perfect (above 0.81), substantial (0.61–0.80), good (0.41–0.60), moderate (0.21–0.40) or slight (below 0.2). The κ values and the 95% confidence intervals (95%CI) were calculated using the online VassarStats statistical tool (<http://vassarstats.net/kappa.html>).

3. Results

3.1. Description of data from pooled serum testing

A total of 49,440 pools from 6216 BoHV1-free certified herds and 1122 pools from 82 infected herds were tested by the IDEXX indirect ELISA in seven OL. Five distinct OL used the IDVet indirect ELISA to analyze 8294 pools from 1218 BoHV1-free certified herds and 744 pools from 75 infected herds. The number and size of pools greatly varied between herds since the pooled sample strategies were based on both the number of samples per herd and the laboratory costs. The maximum pool size was 10 sera, as defined in the analytical process for BoHV1 surveillance in France (Fig. 1A). Pools of size 10 were used in 3319 (44%) herds analyzed by pooled sample testing. Pool sizes up to 9, 8 and ≤ 7 were performed in 980 (13%) herds, 2692 (35%) herds and 600 (8%) herds, respectively. As shown in Fig. 1B, the proportions of pools of size 10 and 9 were comparable for the two pooled serum ELISAs (25% and 22% for IDEXX, and 46% and 38% for IDVet, respectively). In contrast, pools of size 8 were overrepresented in IDEXX testing, in comparison with IDVet (39% vs 8%, respectively), due to the fact that one OL only used pools of size 8. In BoHV1-free certified herds, the number of positive pools was 1575 (3.2%) and 205 (2.5%) for IDEXX and IDVet, respectively. They were distributed in 1068 (17%) and 180 (15%) herds tested by IDEXX and IDVet, respectively. Among these herds, 974 (78%) herds contained a single positive pool. For comparison, the number of positive pools in infected herds were 302 (26.9%) and 252 (33.9%) for IDEXX and IDVet, respectively, with an average number of 3.5 positive pools per herd.

3.2. Individual status of sera composing positive pools

Results of confirmatory tests by gB/gE blocking ELISAs performed at the OL on individual sera composing the positive pools are summarized in Table 1. For BoHV1-free certified herds, the proportion of positive pools which were not confirmed by gB individual testing (false positive pools) did not significantly differ according to the pool sizes and represented 2.4% (1194 pools) and 2.3% (193 pools) of the total number of pools tested by IDEXX and IDVet, respectively. On this basis, the relative diagnostic specificity of pooled serum testing was 97.6% (95%CI: 97.4–97.7%) and 97.7% (95%CI: 97.3–98.0%) for IDEXX and IDVet indirect ELISAs, respectively. False positive pools were distributed in 926/6216 (14.9%) herds tested by IDEXX and 171/1218 (14%) herds tested by IDVet, leading to a herd relative specificity of 85.1% (95%CI: 84.2–86.0%) and 86.0% (95%CI: 83.9–87.8%), respectively. Positive pools containing only gB+/gE- samples were observed for the two kits in both types of herds (infected and BoHV1-free). They were distributed in 363/7434 (5%) BoHV1-free certified herds and 23/157 (15%) infected

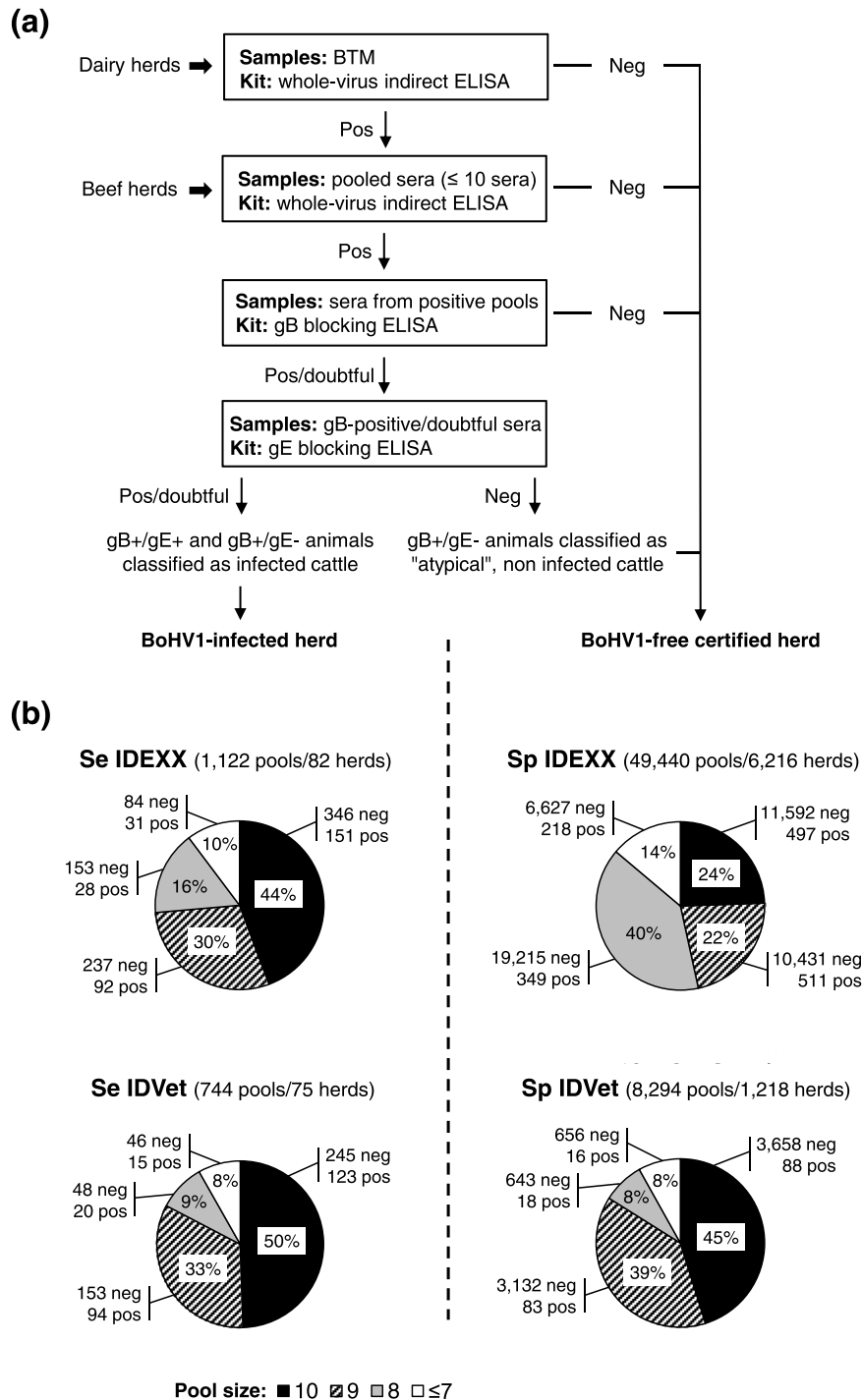


Fig. 1. Results of pooled serum testing in the frame of the BoHV1 surveillance campaign 2018–2019 in 12 French departments. (A) Flow chart of the analytical process according to the BoHV1 surveillance in France. (B) Size, number and serological status of pools used in this study for the evaluation of the relative sensitivity (Se) and specificity (Sp) of the IDEXX and IDVet pooled serum ELISAs.

herds. Their proportion was significantly higher ($p < 0.005$) in infected herds than in BoHV1-free certified herds (1.6% [95%CI: 1.01–2.52%] vs 0.8% [95%CI: 0.70–0.85%] for IDEXX and 2.2% [95%CI: 1.33–3.55%] vs 0.1% [95%CI: 0.08–0.25%] for IDVet). Among these positive pools, 353/399 (88.5%) pools tested by IDEXX and 19/28 (67.9%) pools tested by IDVet contained a single gB-positive sample, including pools of size 10. As expected, no gB+/gE+ samples were detected from positive pools in BoHV1-free certified herds. In infected herds, the gB+/gE+ samples were retrieved from 262/302 (86.8%) and 229/252 (90.9%) positive pools tested by IDEXX and IDVet, respectively. The number of gB+/

gE+ samples in positive pools according to the pool size and the within-herd prevalence are depicted in Fig. 2. Pools of size 10 containing a single gB+/gE+ sample were found in 56/122 (46%) infected herds (Panel a). Remarkably, most of these herds (46/56) showed a BoHV1 seroprevalence below 10%, and 24 of them contained no more than one infected animal. In the other herds, BoHV1 infection was detected through pools of size 10 containing at least two gB+/gE+ samples, or by pools of smaller sizes (Panels b and c, respectively). In most of cases, the absence of pools of size 10 containing a single gB+/gE+ sample could be explained by either a high BoHV1 seroprevalence (Panel b) or a low

Table 1

Composition of positive pools from BoHV1-free certified and infected herds. Sera from positive pools were tested individually by gB blocking ELISA. Non-negative gB samples were retested by gE blocking ELISA.

Herd status	Indirect ELISAs	Pool sizes	Total number of pools	Composition of positive pools ^a			
				gB-	gB+/gE-	gB+/gE+	
BoHV1-free	IDEXX	10	12,089	362	135	0	
		(6216 herds)	9	10,942	387	124	0
		8	19,564	273	76	0	
		≤ 7	6845	172	46	0	
	Total	49,440	1194	381	0		
	IDVet	(1218 herds)	10	3746	85	3	0
		9	3215	75	8	0	
		8	661	17	1	0	
		≤ 7	672	16	0	0	
	Total	8294	193	12	0		
Infected	IDEXX	(82 herds)	10	497	14	8	129
		9	329	4	3	85	
		8	181	1	4	23	
		≤ 7	115	3	3	25	
	Total	1122	22	18	262		
	IDVet	(75 herds)	10	368	3	7	113
		9	247	3	9	82	
		8	68	0	0	20	
		≤ 7	61	1	0	14	
	Total	744	7	16	229		

^a Positive pools were classified according to the results of individual serum testing.

proportion of pools of size 10 combined with a low prevalence (Panel c).

3.3. Individual testing of sera composing negative pools from infected herds

To determine whether pooled serum ELISAs could yield false negative results, gB/gE individual testing was performed on sera composing all negative pools from a subset of 75 infected herds, including 52 herds tested by IDEXX ELISA (676 pools) and 23 herds tested by IDVet ELISA (203 pools). As shown in Table 2, false negative pools ($n = 43$) were observed for the two kits, whatever the pool sizes. They represented 4.1% and 7.4% of negative pools tested by IDEXX and IDVet ELISAs, respectively. All of them contained a single gB+/gE- sample reacting in the doubtful range of the gB blocking ELISA in half of cases (data not shown). No gB+/gE+ sample was retrieved from negative pools. False negative pools were distributed in 17/52 (33%) and 12/23 (52%) herds tested by IDEXX and IDVet ELISAs, respectively.

3.4. Agreement between pooled serum ELISAs and individual testing

The concordance of results between pooled serum testing and gB/gE individual testing was evaluated on the basis of negative and positive pools from infected herds (Table 3). The agreement between pooled serum ELISAs and the gB blocking ELISA was very high (94.9% and 95.2% for the IDEXX and IDVet kits, respectively). Since all gB+/gE+ samples were included into positive pools, the agreement between pooled serum ELISAs and the gE blocking ELISA increased to 97.6% and 98.4% for the IDEXX and IDVet kits, respectively. Assuming that only gB+/gE+ samples could be unambiguously classified as true positive, the two pooled serum ELISAs displayed, in field conditions, a relative diagnostic sensitivity of 100% (95%CI: 93.8–100.0% and 93.4–100.0% for IDEXX and IDVet, respectively).

3.5. Reliability of pooled serum ELISAs

As shown in Fig. 2, 118/157 (75%) herds exhibited a BoHV1 seroprevalence below 10%, highlighting the high probability of occurrence of pools of size 10 incorporating a single positive sample. However, only 39% of these weakly infected herds (31/66 herds by IDEXX and 15/52 herds by IDVet) were detected through pools of size 10 containing a single gB+/gE+ sample. In addition, few pools of size 10 (9 pools tested by IDEXX and 2 pools tested by IDVet) contained a single, weak positive sample (data not shown). This distribution of positive samples into pools impeded an accurate estimation of the reliability of pooled serum testing. In order to estimate the reliability of the two pooled serum ELISAs as well as their ability to detect weak positive samples, 100 gB+/gE+ sera from a subset of 61 herds were analyzed individually by pools of size 10. In these herds, the seroprevalence did not exceed 10%, with a number of positive samples of 1 for 29 herds, 2 for 11 herds and ≥ 3 for 21 herds. The selected samples included 70 gE-strong positive sera and 30 gE-weak positive sera. As shown in Table 4, 29/29 and 25/29 singleton positive samples tested positive by pools of size 10 with IDEXX and IDVet ELISAs, respectively. Positive pools were obtained with the two kits for the remaining 32 herds containing more than one positive sample. Seven samples were not detected by pools of size 10 (3 sera by IDEXX and 6 sera by IDVet), all of them corresponding to gE-weak positive sera. These results indicated that at least one gB+/gE+ sample in 61/61 (100%) and 57/61 (93.4%) infected herds could be detected by pools of size 10 with the IDEXX and IDVet ELISAs, respectively.

4. Discussion

The whole-virus indirect ELISA based on serum pooled samples has been recognized as an official method by the new Animal Health Law (Regulation 2016/429/EC) for the surveillance of IBR in cattle farms. The method should be enough sensitive to detect one weak positive sample in the pool. As the lack of specificity is a main concern in the context of a large-scale surveillance, the method should also be enough specific to limit the inherent cost of confirmatory individual analyses. In this study, we provided for the first time estimates of the performance of pooled serum testing through a retrospective analysis of data from the IBR surveillance campaign 2018–2019 in France. Our results showed that the diagnostic specificity of the IDEXX and IDVet pooled serum indirect ELISAs was high ($> 97.5\%$), when compared to individual testing by gB blocking ELISA. However, since only one false positive pool was observed in most of BoHV1-free certified herds, the herd specificity of IDEXX and IDVet ELISAs was 85.1% and 86.0%, respectively. Analysis of the composition of all negative and positive pools from a subset of 75 infected herds revealed a concordance of results higher than 94% and 97% between pooled serum indirect ELISAs and individual testing by gB and gE ELISAs, respectively. This slightly increase of agreement was explained by the fact that some gB+/gE- samples were retrieved from negative pools, while all gB+/gE+ samples were found in positive pools, leading to a relative diagnostic sensitivity of 100% in field conditions. Remarkably, 75% of infected herds detected as positive by pooled serum testing during the surveillance campaign had a seroprevalence lower than 10%, and half of these herds contained only one seropositive animal. For comparison, previous studies showed that bulk milk testing was not sufficiently sensitive for reliable detection of infected herds with prevalence lower than 10% (Frankena et al., 1997; Hartman et al., 1997; Nylin et al., 2000; Raaperi et al., 2010).

Our results indicated that, in addition to the number of positive sera into pools, the individual level of reactivity had an influence on the pool detection rate. Indeed, all false negative pools of size 10 were composed of a single gB+/gE- sample, most of them (67%) yielding doubtful results by gB ELISA. The true status of animals showing a gB+/gE- serological profile remains questionable. BoHV1-infected animals may be transitory found gB positive and gE negative, particularly at the early

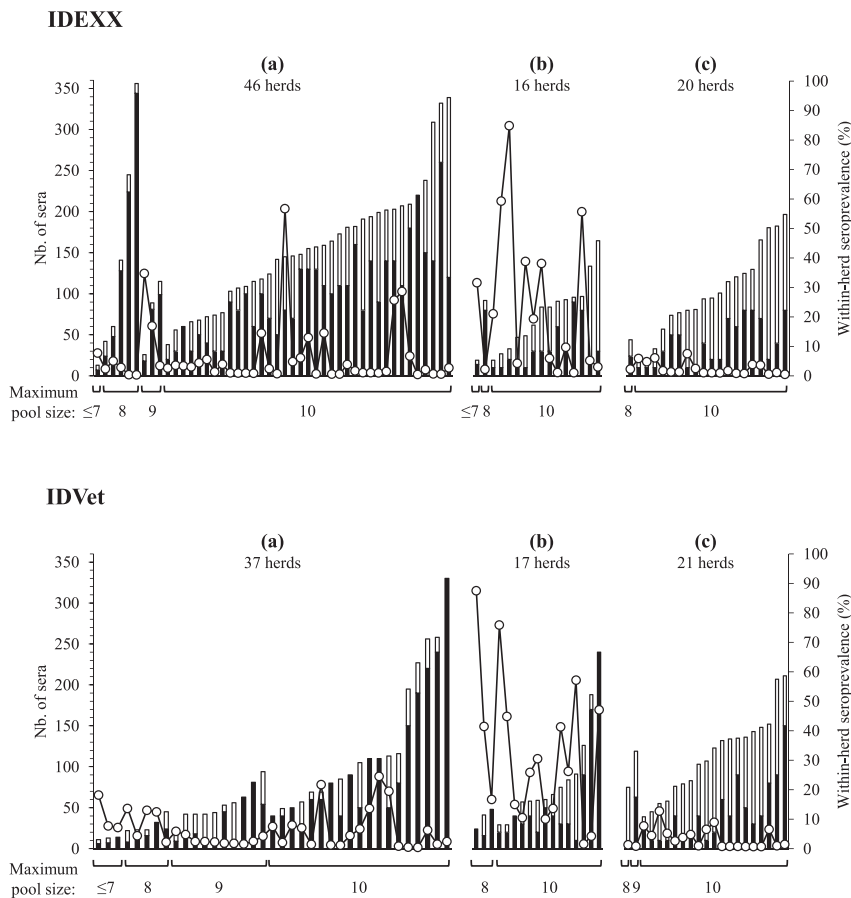


Fig. 2. Number and distribution of BoHV1-positive samples into pools from infected herds. The proportion of samples included into pools of maximum size (black area) as well as the apparent seroprevalence (% of gB+/gE+ samples) are shown for each herd. The maximum size of pools per herd ($\leq 7, 8, 9$ or 10) is indicated below the panels. Panel (a) includes herds for which at least one positive pool of maximum size contained a single positive sample. Panel (b) represents herds for which positive pools of maximum size only contained more than one positive sample. Panel (c) comprises herds for which pools of maximum size did not contain positive samples.

Table 2
Composition of negative pools from a subset of 75 infected herds. Sera from negative pools were tested by gB/gE individual testing.

Indirect ELISAs	Pool sizes	Composition of negative pools ^a		
		gB-	gB+/gE-	gB+/gE+
IDEXX (52 herds)	10	289	19	0
	9	184	5	0
	8	126	2	0
	≤ 7	49	2	0
	Total	648	28	0
IDVet (23 herds)	10	102	8	0
	9	69	3	0
	8	9	3	0
	≤ 7	8	1	0
	Total	188	15	0

^a Negative pools were classified according to the results of individual serum testing.

Table 3
Concordance of results between pooled serum testing and individual testing in BoHV1-infected herds.

Tests	Pools with positive samples	Pools without positive samples	Agreement ^a	False positive	False negative	κ
IDEXX Pool/gB	308	670	94.9%	3.3%	9.1%	0.8809
IDEXX Pool/gE	262	670	97.6%	3.3%	0.0%	0.9431
IDVet Pool/gB	260	195	95.7%	3.6%	5.8%	0.9018
IDVet Pool/gE	229	195	98.3%	3.6%	0.0%	0.9667

κ : Cohen's kappa coefficient.

^a The agreement is expressed as the proportion of concordant results (negative and positive results) between pooled serum testing and gB/gE individual testing. All gB-negative sera were considered to be gE-negative.

stage of infection (de Wit et al., 1998). This is explained by an early and vigorous production of antibodies directed against the gB protein (Kramps et al., 1994; Babiuk et al., 1996; König et al., 2003), combined with a higher sensitivity of gB ELISA on individual serum samples, compared with the gE ELISA (Perrin et al., 1996; Beer et al., 2003; Kramps et al., 2004; Tignon et al., 2017). Otherwise, antigenic cross-reactivity with BoHV1-related herpesviruses may be responsible for nonspecific reactions by indirect and gB ELISAs (Böttcher et al., 2012; Valas et al., 2019; Petrini et al., 2020), whereas the gE ELISA appeared to be very specific (Van Oirschot et al., 1997; Böttcher et al., 2012; Tignon et al., 2017; Valas et al., 2019). We assumed that gB+/gE-samples in BoHV1-free certified herds corresponded to false positive reactions because serological control of herds (every three months for dairy farms and annually for beef farms) greatly exceeds the seroconversion window, rendering unlikely the occurrence of gB+/gE- profiles without any gE positive reaction. Moreover, next annual surveillance controls failed to provide any sign of BoHV1 infection in herds with only gB+/gE- reactions (data not shown). Positive pools exclusively composed of gB+/gE- samples were distributed in 15% and 5% of

Table 4

Detection of low BoHV1 within-herd seroprevalences by pools of size 10. A panel of 100 gB+/gE+ samples from 61 infected herds with low BoHV1 seroprevalence ($\leq 10\%$) were tested individually in reconstituted pools of size 10 by the two pooled serum ELISAs.

Within-herd prevalences (Min-Max)	Nb. of gB+/gE+ samples per herd	Nb. of sera tested ^a	Nb. of herds	Nb. of positive pools		Nb. of positive herds ^b	
				IDEXX	IDVet	IDEXX	IDVet
0.3–1.4%	1	29	29	29	25	29	25
0.6–3.3%	2	18	11	17	17	11	11
1.4–10.0%	≥ 3	53	21	51	52	21	21

^a No more than three gB+/gE+ sera per herd were tested by pooled serum ELISAs. Each serum was tested individually by pools of size 10. Some sera were not available.

^b Herds for which at least one pool of size 10 yielded a positive result.

infected and BoHV1-free certified herds, respectively, and their proportion was significantly higher in infected herds. Although it could not be ruled out that BoHV1 infection increased the susceptibility of cattle to other herpesviruses cross-reacting with diagnosis of IBR, it was likely that some gB+/gE- samples in infected herds represented animals at an early stage of seroconversion. Taken into account these findings, positive pools containing gB+/gE- samples were excluded for estimation of the relative diagnostic specificity by using data from BoHV1-free certified herds, whereas negative pools containing gB+/gE- samples were considered as false negative pools for determination of the agreement with individual testing by using data from infected herds.

The use of surveillance data for estimation of the performance of pooled serum testing displays two main benefits compared with standardized protocols based on reconstitution of pools by using a collection of individual samples. Firstly, surveillance data compile results of thousands of pools from many herds in different geographic regions, ensuring an accurate evaluation of the diagnostic performance. Secondly, surveillance campaign for IBR requires testing of all cattle older than 24 months for most of beef herds, and all lactating cows in dairy herds with a positive bulk milk testing. This allows the measurement of the full range of sample reactivity at the herd scale and the identification of the threshold level of BoHV1-specific antibodies (the so-called weak positive samples) that should be detected by screening tests. In contrast, field sample collections often represent a fraction of animals in each herd, and positive samples tend to be strong positive samples since collection mainly targets animals which were detected positive several weeks or months ago. Inversely, the estimation of the performance of pooled serum testing using surveillance data presents two main drawbacks in comparison with pools created with reference samples. Sampling in a given herd can be done in a sequential manner (different days) and pooling strategies may differ between official laboratories. Consequently, pool size is not uniform and can vary both at the herd level and between herds. Furthermore, sequential sampling combined with some management practices such as grazing of cattle groups on different pasture lands, may lead to the distribution of negative and positive samples in pools in a non-stochastic manner, limiting the evaluation of the sensitivity. These limitations led us to test separately positive samples from a subset of infected herds in reconstituted pools of size 10 in order to mimic the worst-case scenario. The results revealed that pooled serum ELISAs failed to detect some gE-weak positive samples. This diagnostic drawback was counteracted at the herd level for the IDEXX ELISA since at least one positive sample in each infected herd yielded positive result when tested alone in a pool of size 10. The IDVet ELISA showed a lower herd diagnostic sensitivity (93.4% vs 100%) due to the fact that some singleton positive samples were undetected by pools of size 10.

5. Conclusion

Pooled serum testing has been recently approved under the European Union Animal Health Law for serological surveillance of IBR in non-vaccinated bovine population. This cost-effective strategy is challenging since partitioning of sera into pools requires the detection of a

single weak-positive sample in the pool to prevent diagnostic failure. Application of pools of size 10 in both field and experimental conditions highlighted the ability of pooled serum testing to detect a wide range of BoHV1 singleton reactors, including weak-positive samples. However, in the case of ongoing infection, cattle at a very early stage of infection might be undetected. In the French monitoring program, sera composing negative pools in herds showing positive reactions by gB and gE ELISAs are also controlled by individual testing to ensure eradication of all infected animals. Inversely, in non-infected herds, pooled serum testing is a convenient method to reduce false-positive results by individual testing due to antigenic cross-reactions with BoHV1-related herpesvirus.

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CRedit authorship contribution statement

SV, DN-M and SM designed the study. DN-M and SM facilitated the collection of epidemiological data. SS performed the laboratory analyses. SV performed the data analyses and wrote the manuscript. MT, SM and DN-M revised the manuscript critically. All co-authors read and approved the final manuscript.

Conflict of interest statement

All authors deny any financial and personal relationships with other people or organizations that could inappropriately influence this work.

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