Surveillance of Infectious Bovine Rhinotracheitis in marker-vaccinated dairy herds: Application of a recombinant gE ELISA on bulk milk samples

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Surveillance of Infectious Bovine Rhinotracheitis in marker-vaccinated dairy herds: application of a recombinant gE ELISA on bulk milk samples

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ABSTRACT

Infectious Bovine Rhinotracheitis (IBR) occurs worldwide, requiring significant resources for eradication programs or surveillance purposes. The status of infection is usually detected by serological methods using the virus neutralization test (VNT) or enzyme-linked immunosorbent assay (ELISA) on individual sera. The gE DIVA (Differentiating Infected from Vaccinated Animals) vaccines approach, adopted in order to reduce the virus circulation and prevent clinical signs, have tightened the range of available methods for the serological diagnosis. Different gE blocking ELISA could be performed to detect specific antibodies in sera of infected or whole virus-vaccinated animals but with less sensitivity if applied to bulk milk samples, especially in marker-vaccinated herds. A new rec-gE ELISA was recently developed in Italy and applied with good performances on blood serum samples. The present paper focuses on the application of a rapid protocol for purification/concentration of immunoglobulin G (IgG) from bulk milk and on the use of the new rec-gE indirect ELISA. The study involved three different partners and 225 herds (12800 lactating cows) with different official IBR diagnostic statuses. The diagnostic specificity of the method was demonstrated closed to 100% while the diagnostic sensitivity was strictly related to the herd-seroprevalence. Considering 2.5% as the limit of detection of within-herd seropositivity prevalence, the diagnostic sensitivity showed by the proposed method was equal to 100%. A single reactivation of a whole strain vaccine in an old cow was detected inside a group of 67 lactating cows, showing the field applicability of the method.

INTRODUCTION

Infectious Bovine Rhinotracheitis (IBR) is a widespread animal disease caused by Bovine Herpesvirus (BoHV-1) type 1, with a substantial impact on animal health and livestock productivity (Raaperi et al., 2014). Upon primary infection, BoHV-1 replicates in the mucous membranes of either the respiratory or the genital tract. From there, it will gain access to local sensory neurons for establishment of latency in the corresponding ganglia, reactivating itself in the presence of stress, immunosuppression or treatments with corticosteroids (Ackermann and Engels, 2006; Winkler et al., 2000).

DIVA-vaccines, based on BoHV-1 glycoprotein E (gE) deleted strain, have been reported to be a safe and effective strategy against IBR that can be successfully used in countries with high prevalence of infection (Kaashoek et al., 1995).

Currently, different diagnostic tools are suitable for the surveillance of BoHV-1 infection. Evidences of viral circulations can be detected using the gold standard virus neutralization test (VNT), or indirect ELISAs based on whole-virus antigens. Diagnostically, specific ELISA protocols must be performed in herds where marker vaccines are used, to correctly differentiate between infected and vaccinated animals. Because gE deleted vaccines represent the most commonly used approach (Mars et al., 2001; Rijsewijk et al., 1999), serological investigations are based on the combination of a whole-virus-based indirect ELISA or a glycoprotein B (gB) based competitive ELISA with a gE -based competitive ELISA. An indirect ELISA test was recently developed based on the identification of anti BoHV1-gE antibodies (Bertolotti et al., 2015). In all previously mentioned cases the diagnostic approaches are based on blood serum and on the individual testing of the whole herds.

Bulk milk (BM) can represent a readily available sample matrix for screening tests. Indeed, the BM sampling procedure is non-invasive for the animals, avoiding the negative effects, such as stress, that an invasive blood sampling can cause (Reber et al., 2012); moreover, the lower expenses for farm visits, blood sampling procedures and necessary materials identify it as a valuable and inexpensive sampling method (Reber et al., 2012).

In Countries or regions where IBR eradication has already been achieved, surveillance can be easily performed by indirect ELISA test on BM samples. Unfortunately, if BM samples are tested by competitive gE ELISA when vaccination is still ongoing, IBR diagnosis can feature limited sensitivity (Kramps et al., 2004). Even if individual milk was shown to be a reliable biological matrix for diagnosis of BoHV-1 (Schroeder et al., 2012), blocking ELISAs

87 can prove unable to detect the seroconversion caused by an IBR wild strain infecting a vaccinated herd because of
88 the required dilution of the samples and the lower concentration of antibodies in milk. Some methods for
89 improving the sensitivity of ELISA tests were recently proposed (Schroeder et al., 2012) based on the
90 concentration of immunoglobulins from the milk.
91 In this paper we describe a cost-effective approach for IBR surveillance in vaccinated herds. Immunoglobulins (Ig)
92 in the BM samples are purified, concentrated and subsequently tested with a new indirect ELISA test based on the
93 reactivity against the BoHV-1 gE (Bertolotti et al., 2015). In particular, the recombinant indirect gE ELISA was used
94 in association with a rapid protocol for the concentration and purification of IgG from BM samples. The aim of this
95 study was the evaluation of the performances of this new serological method on samples collected from three
96 Italian Provinces (Turin, Cuneo and Trento).
97
98 **MATERIALS AND METHODS**
99 *Purification and concentration of bulk milk IgG*
100 A protein G (pG) based affinity matrix was prepared following a standard recombinant approach. Briefly, the
101 Streptococcal pG gene subunit, lacking the albumin-binding region, was cloned into pGEX-6His, expressed as
102 GST/pG fusion protein and purified under native condition by immobilized metal ion affinity chromatography
103 (Smith and Johnson, 1988). The purified protein was covalently bound to agarose beads using low density ABT
104 glyoxal resin (Agarose Bead Technologies MADRID, Spain) following manufacturer's recommendation and at 6mg
105 of GST/pG per ml of settled gel ratio. Following bond stabilization, the affinity matrix was washed with PBS and
106 stored as 50% slurry in 25mM phosphate buffer (pH 7.0) 20% ethanol at 4^{o}C.
107 The purification and concentration protocol was applied to each BM adapting an immunoprecipitation protocol,
108 with minor modification (Harlow and Lane, 1988). Briefly, 10ml of BM were subjected to rennet-based casein
109 precipitation. After curd breaking and 5 minutes of incubation on ice, the sample was centrifuged at 3600g for 10
110 minutes at 4°C. Following the separation from lipids and curd, about 6ml of milk whey were decanted into a new
111 tube and incubated with 100µl of 50% affinity matrix for 10 minutes at room temperature under gentle agitation.
112 The adsorbed matrix was centrifuged and the pellet was loaded into a mini spin column. The matrix was washed
113 twice (Tween 0.05%, EDTA 0.5 mM, NaCl 160 mM) and the IgG were eluted in 200µl of 0.1M glycine buffer pH 2.8

onto a collection tube preloaded with 1/10 volume of Tris 1M, pH 8.2. A Bradford quantification assay was performed to confirm presence of at least 1.5 mg/ml of IgG in the purified/concentrated (PC) eluted sample, using Bovine Gamma Globulin (BIORAD Quick Start) standard curve. Only samples with a sufficient IgG concentration have been considered suitable for testing in the indirect ELISA. Moreover, the effectiveness of purification was initially confirmed in a consistent subset of samples by SDS-PAGE.

Indirect gE ELISA

In a previous work, we developed and validated an indirect ELISA assay based on the reactivity of bovine sera against BoHV-1 glycoprotein E (gE) expressed in recombinant form. Briefly the ectodomain of gE was cloned into a mammalian expression vector and expressed as secreted protein in protein free medium as described (Bertolotti et al., 2013). The test showed very good performances, especially in terms of specificity. Compared to the original protocol, the ELISA assay was slightly modified and adapted to milk samples. Briefly, each sample, represented by purified/concentrated bulk milk (PC-BM) was diluted 1:2 directly in plate in PBS 1.25% casein and placed into two adjacent wells, the first coated with the recombinant BoHV-1 gE while the second one with a negative antigen. The plates were incubated at room temperature for 60 minutes. After four washing cycles, a peroxidase labeled secondary antibody, diluted at 10 ng/ml in PBS 1.25% casein, was added to each well. After 45 minutes of incubation at room temperature, four washing cycles were performed before the addition of the substrate solution (3,3',5,5'-tetramethylbenzidine, TMB). The reaction was stopped with 0.2M H_2SO_4 after 15 minutes at room temperature, and the absorbance value determined at 450 nm. The net reactivity (net optical density, or OD) was calculated for each sample as the difference between the absorbance of the gE and negative antigen wells. The results were expressed as the percentage of reactivity against the net OD of the positive control included in each plate.

Considering that samples represent a pool of individual milks, we modified the cut-off of the test compared to the previous work as suggested (Böttcher et al., 2012). The samples with a reactivity percentage greater than 40% were classified as positive, those with reactivity percentage between 30% and 40% as doubtful, and those with a reactivity percentage lower than 30% were considered negative.

Evaluation of analytical and diagnostic performances

A selection of 7 individual milk samples belonging to IBR-positive farms was used to evaluate the efficacy of IgG purification/concentration method in rec-gE ELISA protocol. The purified/concentrated milk samples were obtained as described above. Twofold dilution of each sample was tested by rec-gE ELISA and compared to the reactivity of the corresponding untreated milk. The yield was defined as the dilution showing the same reactivity of the untreated sample.

The limit of detection of the proposed method was estimated using spiked samples obtained diluting an IBR positive individual milk in increasing number of negative individual milks. Each test was performed in triplicate. Sensitivity and specificity values were calculated considering the official IBR status as the gold standard and the 95% confidence intervals of these parameters were calculated by using Exact Binomial test with R statistical software (R Core Team, 2015).

Milk samples

Bulk milks were collected from dairy herds of three Italian Provinces (Turin, Cuneo and Trento), where IBR surveillance and eradication programs have been in effect since 2004 (Turin and Cuneo) and 2002 (Trento). Milk samples included a total of 225 dairy farms covering all different official IBR statuses.

Three different sets of BM were used in order to evaluate the performances of the proposed diagnostic protocol. Details about farm numbers and IBR statuses are reported in Table 1.

The Department of Veterinary Science of the University of Turin (DVS) conducted a preliminary validation based on laboratory tests and field data. The field sample set included BM collected from 76 herds belonging to the Turin province corresponding to 4784 lactating cows. In case of unexpected results (i.e disagreement with official IBR status) further analyses were conducted at individual level, including milk and blood sampling.

A larger field application was conducted in the second part of the study, involving three different partners: the Department of Veterinary Sciences of the University of Turin (DVS), the Official Diagnostic Laboratory *Istituto Zooprofilattico Sperimentale di Piemonte Liguria e Valle d'Aosta* (IZSPLV) in Turin, and the *Istituto Zooprofilattico Sperimentale delle Venezie* (IZSVe) in Legnaro. A total of 149 farms were included in this phase, for a total of 8016 lactating cows.

In all cases data about the farm's official IBR status (evaluated at the same time of milk collection or based on the most recent official serological investigation) and the number of lactating cows were collected. Unfortunately, the ear tag identification numbers, useful for the estimation of seroprevalence, were available only during the preliminary validation.

RESULTS

Evaluation of analytical sensitivity

The reactivity of the PC milks was compared to the reactivity of the corresponding untreated sample. In all seven cases the PC milks diluted 1:16 showed higher reactivity than the untreated one (fig. 1). The average yield of the PC protocol was identified as 27.3x. On the other hand, spiked samples are classified as IBR positive if positive individual milk is diluted in 39 negative samples. This result suggests the theoretical prevalence of 2.5% in lactating cows as detection limit (i.e. diagnostic sensitivity) of the method (fig. 2).

Field application

The first set of PC-BM were collected from 76 herds from the Turin province (30 IBR-positive herds, 29 IBR-marker-vaccinated farms, and 17 to IBR-free farms).

The results for the positive herds were strictly dependent on the intra-herd prevalence (Fig. 3).

Among the 30 IBR-positive farms, 14 have an estimated internal prevalence lower than 2,5%: thirteen of them resulted negative while a single herd, whose data on the intra-herd prevalence had not been updated, showed full-scale reactivity due to a recent BoHV-1 viral circulation. Of the remaining 16 positive herds, 15 PC-BM showed a positive outcome in the rec-gE ELISA test, reflecting the true infectious status; just one farm, where a few animals had been vaccinated many years before using a whole-virus strains, resulted negative.

Regarding the 29 IBR-marker-vaccinated farms, all their PC-BM showed negative outcomes in the rec-gE ELISA.

Among the 17 farms classified as IBR-free, 16 PC-BM were tested as negative, confirming their negative serological status for BoHV-1 gE. The case of the remaining herd that recently acquired "IBR-free" status was more interesting and further investigated, because its PC-BM showed full-scale reactivity in the rec-gE ELISA test. A new BM and 67 individual milk samples were collected a month later and tested by rec-gE ELISA. The new PC-

BM confirmed the first positive outcome. Only a single individual milk sample showed a high reactivity, while the remaining milks were negative. Epidemiological investigation, as well as individual blood serum tests (IDEXX IBR gE ab ELISA), confirmed a seroconversion in this animal due to reactivation of whole-virus vaccine administered more than 10 years before (fig. 4).

A second sample set included BM from 86 farms belonging to the provinces of Turin and Cuneo and 63 from the province of Trento.

Among the positive farms (n=21), 3 samples resulted negative. Further investigations confirmed a seroprevalence lower that 2.5% among lactating cows in two farms, and the presence of a single whole-virus vaccinated animal in the last farm.

Only 2 out the 128 expected negative farms showed a positive results in rec-gE ELISA. In both cases, the BM included a very small number of lactating cows (5 and 9 animals respectively).

Evaluation of diagnostic specificity and sensitivity

Performances of the proposed method were evaluated. Diagnostic specificity of the test was high (dSp = 98.85% 95CI: 95.90% - 99.86%) whereas the diagnostic sensitivity is strictly related to the seroprevalence in lactating cows. As expected, the antibodies of few positive animals diluted in a large number of negative milks are not always detectable by the proposed test. However, when antibody titer increases because of a viral circulation as well as a whole virus vaccine reactivation, BM can be a very good sample for IBR surveillance. Indeed, the 2.5% as limit of prevalence was previously suggested as possible limit of detection in IBR positive farm (Casarin et al., 2016), field data suggested good performances of the test if used for surveillance purposes in gE negative herds. However, considering 2.5% as the limit of detection of within seropositivity prevalence (Casarin et al., 2016), the diagnostic sensitivity showed by the test is equal to 100% (95%CI: 89.4% - 100%).

DISCUSSION

Blocking gE ELISAs were demonstrated to be less sensitive if milk is used as diagnostic matrix due to the lower concentration of antibodies (0.6 mg/ml) compared to blood (10 mg/ml) (Król et al., 2010; Marnila and Korhonen, 2002). Indeed, Wellenberg and colleagues demonstrated that a commercial blocking gE ELISA is able to detect a

minimum prevalence of 10-15% in a bulk milk sample (Wellenberg et al., 1998). Obviously, the purification and concentration of IgG from bulk milk can overcome this limitation. Moreover, the use of an indirect ELISA could represent a possible diagnostic solution.

The aim of this study was the evaluation of the performances of a new diagnostic method in the detection of antibodies against BoHV-1 gE in bulk milk samples.

Currently, the directive no. 1964/432/EEC of the European Union allows the use of commercial ELISA for Brucella and Enzootic Bovine Leukosis antibodies research in bulk milk of 100–200 cows. In the case of IBR, according to decision n. 2004/558/EC, to date, the largest bulk pool size of the licensed kits corresponds to 50 cows. This approach can be easily applied to IBR-free farms using BHV-1 ab indirect ELISA (as IDEXX Trachitest Serum Screening Ab Test). In fact, antibody response against several structural proteins may achieve the expected sensitivity and may lead to the detection of a few positive animals. In contrast, the IBR surveillance in marker-vaccinated animals is more complex. The anti-gE response investigated using the blocking ELISA approach can suffer in sensitivity (Kramps et al., 2004) and it may not fit with EU regulations. Moreover we cannot exclude that IgG concentration may negatively affect the blocking ELISA specificity due to the steric hindrance caused by the high antibodies concentration: indeed the role of antibodies hindrance was highlighted in hyper-vaccinated animals tested by blocking ELISA, showing false positive results (Kramps et al., 2004). In those farms IBR surveillance is possible only by individual blood serum testing. In this framework, the proposed approach including the IgG concentration protocol and the rec-gE ELISA can solve these drawbacks and can help in IBR surveillance.

Despite the fact that different Italian regions have undertaken voluntary or compulsory programs to eradicate IBR, the Province of Bolzano is the only one that has achieved this goal. The milk sampling procedures are easier and cheaper than the collection of blood serum. For this reason the method described here could be employed in the programs for the control of vaccinated and IBR-free herds.

In the first validation, we evaluated the field applicability of the method. The ELISA cut-off was slightly modified (40% instead of 80% of the reactivity of positive control) and the method's limit of detection in terms of seroprevalence among the lactating cows was identified as 2.5 % (equivalent to a single infected cow in a pool of 40 animals). Indeed, the sensitivity of the newly proposed method seems to be mainly affected by the within-herd

prevalence. Details on the prevalence of IBR-positive animals within each farm revealed the presence of few positive individuals among the lactating cows and suggested the 2.5% as the seroprevalence limit detected by this method. The same limit in detecting positive animals in milk pool was recently identified (Casarin et al., 2016). No false positive results were obtained in this phase; in contrast, the method was actually able to identify a single vaccine reactivation within a farm officially classified as IBR-free, supporting its helpfulness during IBR surveillance. We further investigated the epidemiological situation of that farm, collecting individual milk samples from lactating cows, detecting a single positive animal in a group of 67. This animal was vaccinated with a live whole-virus in 2003, with a consequent lifelong latency of the vaccine strain in the spinal ganglia. To confirm the seroconversion, a commercial gE blocking ELISA test was performed on blood serum from this animal. The positive outcome was confirmed, as well as the absence of successful virus excretion in the herd, considering that all the remaining 66 animals were still negative to the same test one month after the first positive outcome.
In the second part of the study, out of the 21 IBR-positive farms only three resulted as negative but the lack of detailed information about the IBR status of lactating cows that were part of those pools made it impossible to evaluate the real seroprevalence of those samples. All the vaccinated herds (n=106) were correctly classified. Among the 22 IBR-free herds, only two resulted positive. The two cases were similar both for the number of lactating cows (9 and 5 respectively) and for the presence of a single doubtful milk sample diluted in a limited number of negative samples. We speculated that this combination could lead to false positive results.

Conclusions

Bulk milk sample testing is a non-invasive method that is not stressful for the animals. Moreover, milk samples are taken routinely during milk quality assurance programs, so the use of the same sampling infrastructure would be extremely helpful in providing a cost-effective way of monitoring herd status (Reber et al., 2012). Financial comparison between the two methodologies was possible in Switzerland, where the costs were reduced by 50% without any major impact on the quality of the surveillance program (Reber et al., 2012). One limitation of bulk milk as diagnostic matrix is that it only represents the cattle delivering milk to the bulk tank on the day of sampling, therefore excluding any non-milking heads such as dry cows, diseased cows and cows in the colostral period. The use of milk samples can however increase the flexibility of the surveillance programs, for example by

increasing the intensity of surveillance in case of disease events or increase in the early detection capabilities of the survey design. The main limitation experienced in this work was the impossibility to calculate beforehand the prevalence within each bulk milk sample. To overcome this limit, a further investigation is taking place, through an important collaboration with the sanitary authorities and the Regional farmers association. The evaluation of the ideal pool sample size should be conducted as previously suggested (Schroeder et al., 2012). As demonstrated in this work, pooled milk from few samples is more susceptible to a false positive result in rec-gE ELISA test; conversely milk pools comprising around 40 animals represent the best target sample for this test. A more robust evaluation of the field applicability of this method could be performed, supported by the suggestions described above, to confirm the preliminary limit of detection of 2,5%. Nevertheless, this work represents an important first step towards a validation pathway of an innovative serological assay to detect antibodies against BoHV-1 gE.

Abbreviations

IBR: Infectious Bovine Rhinotracheitis

DIVA: Differentiating infected from vaccinated animals

Ig: immunoglobulins

BM: bulk milk

PC: purified/concentrated

PC-BM: purified/concentrated bulk milk

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298 Table 1. Description of BM collected during the study.

	Laboratory	Positive farms	IBR-free farms	IBR marker vaccinated farms
Preliminary validation	DSV	30	17	29
Second validation	DSV IZSPLV	5	22	59
	DSV IZSVe	16	4	43
		51	**43**	**131**

299

300

Figure 1. Estimation of analytical sensitivity. Mean reactivity of 7 untreated individual milks (nt, white circle) compared to corresponding PC samples (black circles) at different dilutions. Vertical bars: standard deviation.

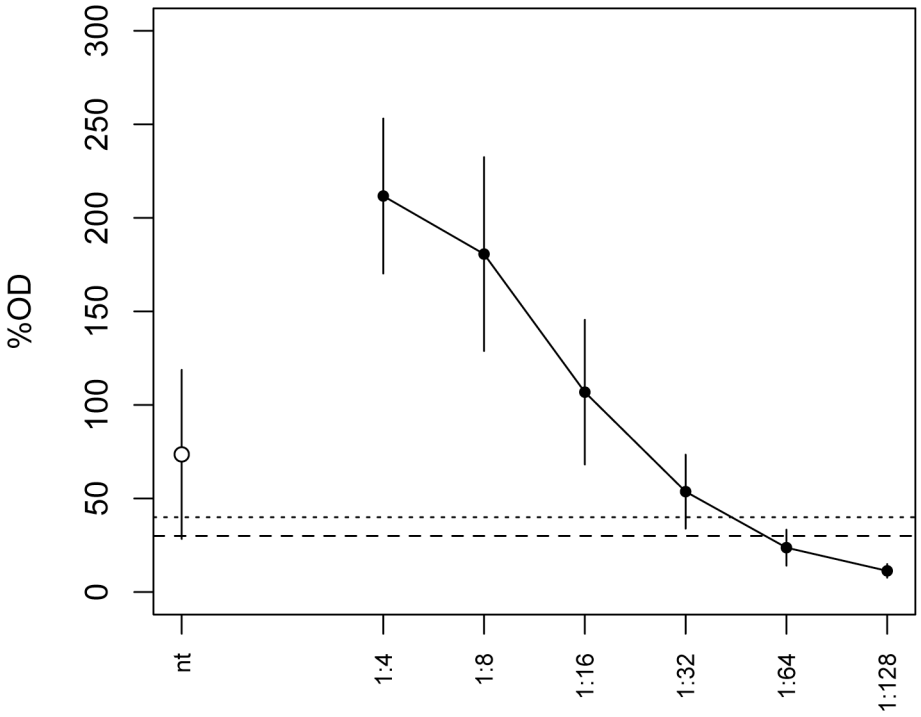


Figure2. Estimation of the analytical sensitivity by testing the reactivity of spiked milks. Individual milk from a IBR positive animal was used to spike different volumes of negative milks (4, 9,19, 29, and 39 volumes). Each sample was tested both untreated and after PC protocol. Gray bars: Untreated spiked milk samples; Black bars: PC spiked milk samples; Horizontal lines represent the cutoff limits.

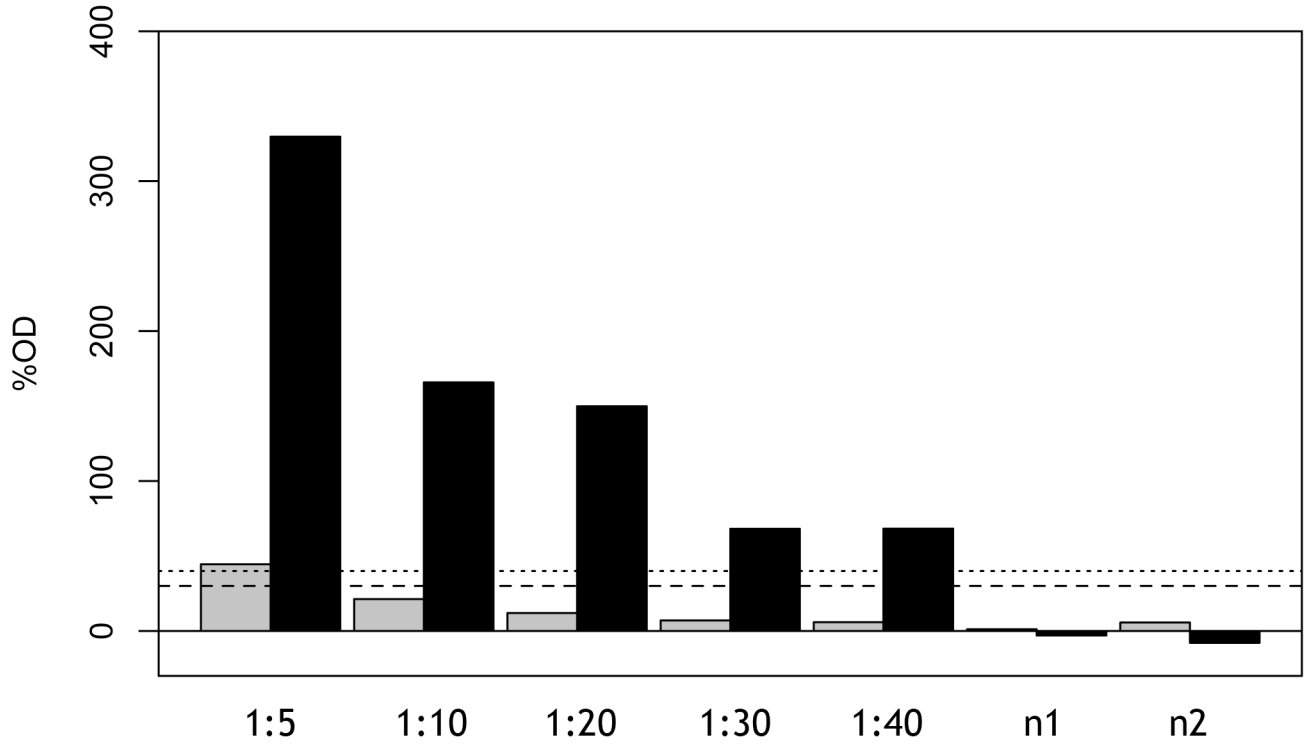


Figure 3. Distribution of rec-gE ELISA results during the preliminary validation. Circles: PC-BM from gE negative farms. Triangles: PC-BM from gE positive farms. Solid-black points represent the three farms that showed unexpected results (see the text for details). Horizontal dashed line: ELISA positivity cutoff. Vertical dotted line: within-herd prevalence of 2.5%.

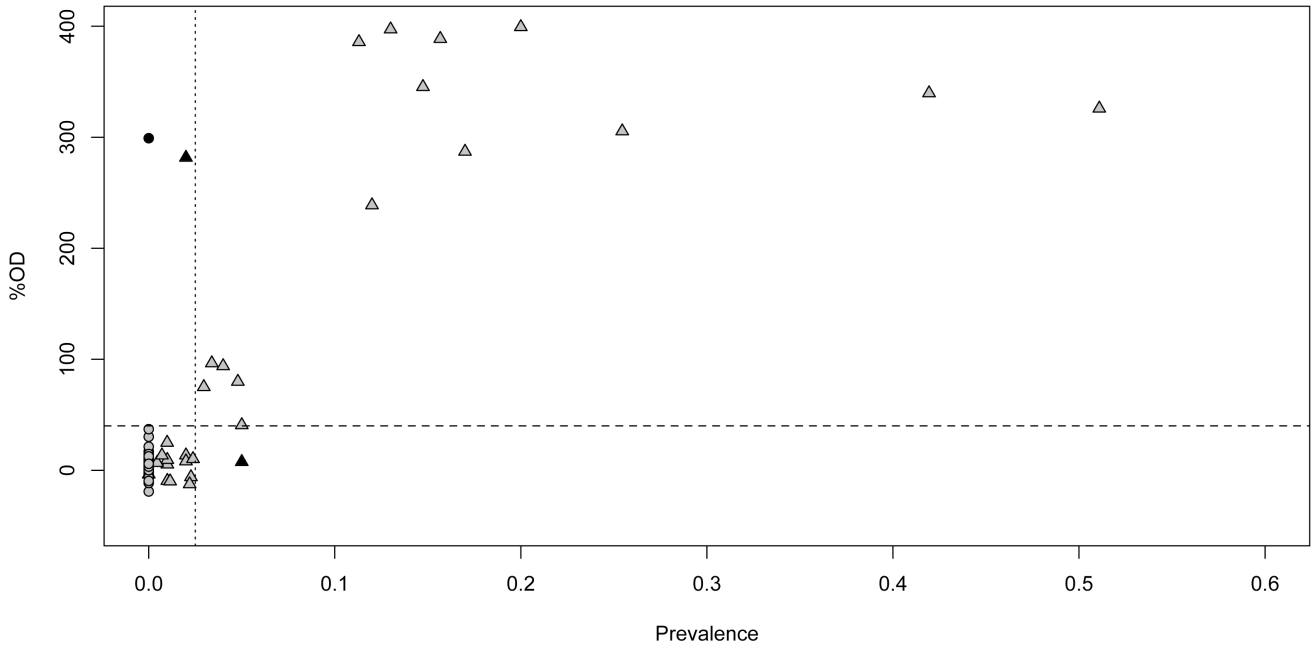
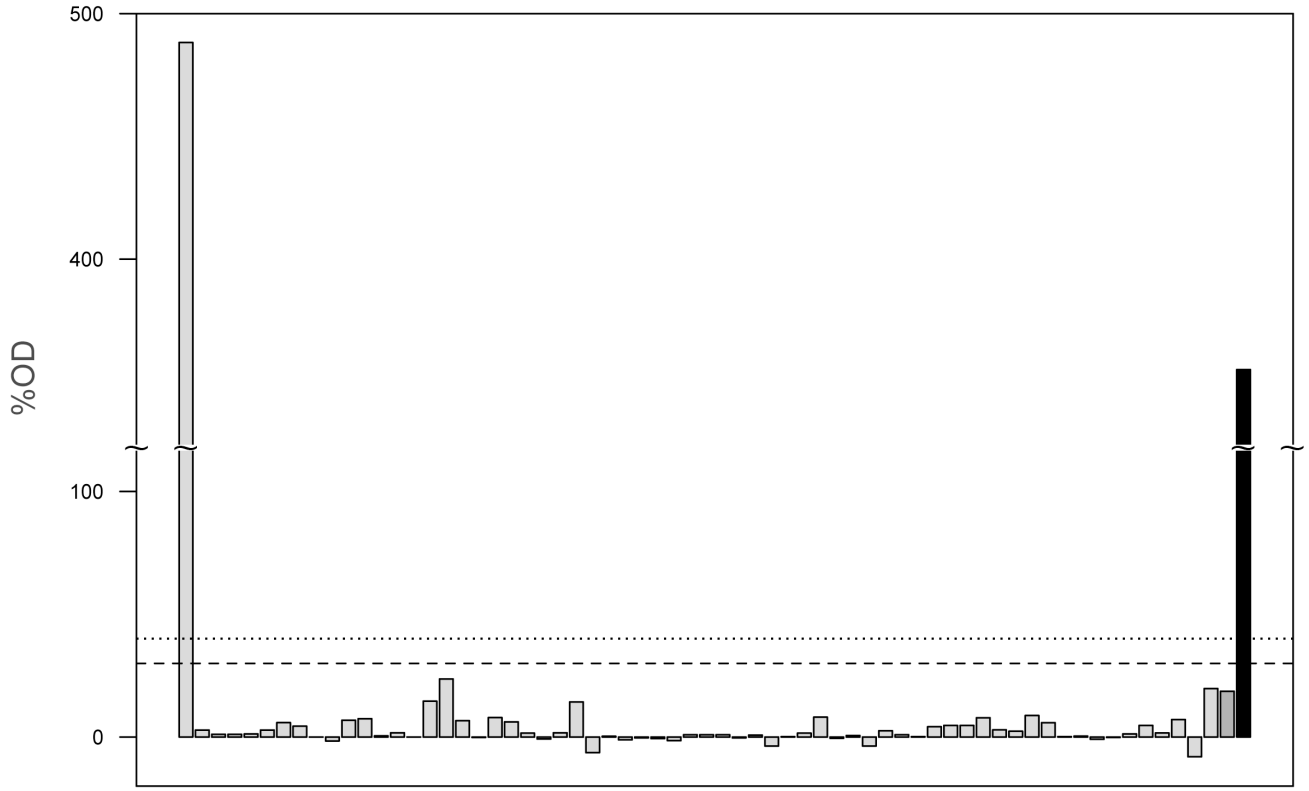


Figure 4. Reactivity of samples from the farm showing vaccine reactivation. White bars: reactivities of the 67 individual untreated milk samples; gray bar: reactivity of the untreated BM; black bar: reactivity of the PC-BM sample. Horizontal lines represent the cutoff limits.



REFERENCES

Ackermann, M., Engels, M., 2006. Pro and contra IBR-eradication. Vet. Microbiol. 113, 293–302. doi:10.1016/j.vetmic.2005.11.043

Bertolotti, L., Muratore, E., Nogarol, C., Caruso, C., Lucchese, L., Profiti, M., Anfossi, L., Masoero, L., Nardelli, S., Rosati, S., 2015. Development and validation of an indirect ELISA as a confirmatory test for surveillance of infectious bovine rhinotracheitis in vaccinated herds. BMC Vet. Res. 11, 300. doi:10.1186/s12917-015-0612-5

Bertolotti, L., Rosamilia, A., Profiti, M., Brocchi, E., Masoero, L., Franceschi, V., Tempesta, M., Donofrio, G., Rosati, S., 2013. Characterization of caprine herpesvirus 1 (CpHV1) glycoprotein E and glycoprotein I ectodomains expressed in mammalian cells. Vet. Microbiol. 164, 222–228. doi:10.1016/j.vetmic.2013.02.008

Böttcher, J., Boje, J., Janowetz, B., Alex, M., König, P., Hagg, M., Götz, F., Renner, K., Otterbein, C., Mages, J., Meier, N., Wittkowski, G., 2012. Epidemiologically non-feasible singleton reactors at the final stage of BoHV1 eradication: serological evidence of BoHV2 cross-reactivity. Vet. Microbiol. 159, 282–290. doi:10.1016/j.vetmic.2012.04.017

Casarin, E., Lucchese, L., Grazioli, S., Facchin, S., Realdon, N., Brocchi, E., Morpurgo, M., Nardelli, S., 2016. A New ELISA Using the ANANAS Technology Showing High Sensitivity to diagnose the Bovine Rhinotracheitis from Individual Sera to Pooled Milk. PloS One 11, e0145912. doi:10.1371/journal.pone.0145912

Harlow, E., Lane, D.P., 1988. Antibodies: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Kaashoek, M.J., Moerman, A., Madić, J., Weerdmeester, K., Maris-Veldhuis, M., Rijsewijk, F.A., van Oirschot, J.T., 1995. An inactivated vaccine based on a glycoprotein E-negative strain of bovine herpesvirus 1 induces protective immunity and allows serological differentiation. Vaccine 13, 342–346.

Kramps, J.A., Banks, M., Beer, M., Kerkhofs, P., Perrin, M., Wellenberg, G.J., Oirschot, J.T.V., 2004. Evaluation of tests for antibodies against bovine herpesvirus 1 performed in national reference laboratories in Europe. Vet. Microbiol. 102, 169–181. doi:10.1016/j.vetmic.2004.07.003

Król, J., Litwińczuk, Z., Brodziak, A., Barłowska, J., 2010. Lactoferrin, lysozyme and immunoglobulin G content in milk of four breeds of cows managed under intensive production system. Pol. J. Vet. Sci. 13, 357–361.

Marnila, P., Korhonen, H., 2002. Immunoglobulins, in: Encyclopedia of Dairy Science. Academic Press, London, UK, pp. 1950–1956.

Mars, M.H., de Jong, M.C., Franken, P., van Oirschot, J.T., 2001. Efficacy of a live glycoprotein E-negative bovine herpesvirus 1 vaccine in cattle in the field. Vaccine 19, 1924–1930.

Raaperi, K., Orro, T., Viltrop, A., 2014. Epidemiology and control of bovine herpesvirus 1 infection in Europe. Vet. J. Lond. Engl. 1997 201, 249–256. doi:10.1016/j.tvjl.2014.05.040

R Core Team, 2015. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Reber, A., Reist, M., Schwermer, H., 2012. Cost-effectiveness of bulk-tank milk testing for surveys to demonstrate freedom from infectious bovine rhinotracheitis and bovine enzootic leucosis in Switzerland. Schweiz. Arch. Für Tierheilkd. 154, 189–197. doi:10.1024/0036-7281/a000329

Rijsewijk, F.A., Verschuren, S.B., Madić, J., Ruuls, R.C., Renaud, P., van Oirschot, J.T., 1999. Spontaneous BHV1 recombinants in which the gI/gE/US9 region is replaced by a duplication/inversion of the US1.5/US2 region. Arch. Virol. 144, 1527–1537.

Schroeder, C., Horner, S., Bürger, N., Engemann, C., Bange, U., Knoop, E.V., Gabert, J., 2012. Improving the sensitivity of the IBR-gE ELISA for testing IBR marker vaccinated cows from bulk milk. Berl. Münch. Tierärztl. Wochenschr. 125, 290–296.

Smith, D.B., Johnson, K.S., 1988. Single-step purification of polypeptides expressed in Escherichia coli as fusions with glutathione S-transferase. Gene 67, 31–40.

Wellenberg, G.J., Verstraten, E.R., Mars, M.H., van Oirschot, J.T., 1998. ELISA detection of antibodies to glycoprotein E of bovine herpesvirus 1 in bulk milk samples. Vet. Rec. 142, 219–220.

Winkler, M.T., Doster, A., Jones, C., 2000. Persistence and reactivation of bovine herpesvirus 1 in the tonsils of latently infected calves. J. Virol. 74, 5337–5346.