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Influence of the incubation temperature and the batch components on the sensitivity of an enzyme-linked immunosorbent assay to detect Aujeszky's disease virus glycoprotein E (gE)

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Summary
Although licensed batches of an enzyme-linked immunosorbent assay (ELISA) for Aujeszky's disease virus (ADV) were used, and the assays were performed within an ISO/IEC 17025 accredited quality control system, certain routine runs of the ADV ELISA were not validated using the quality system criteria, even when all technical parameters were controlled. Incubation at different temperatures and batch composition were identified as parameters that could result in non-validated assays/runs. Therefore, the effect of incubation temperature and batch composition on the analytical sensitivity of the ELISA was investigated. The World Organisation for Animal Health (OIE) standard reference serum ADV1 was diluted 1:8 and tested in 94 different glycoprotein E ELISA runs performed with different batches and different incubation temperatures. The incubation temperature and batch components had a significant influence on the qualitative result for the OIE standard reference serum. An incubation temperature of at least 22°C was recommended, based on the results of this analysis. Which of the batch components caused these differences in sensitivity was not investigated further.

Keywords
Anti-ADV gE enzyme-linked immunosorbent assay – Aujeszky's disease virus – Batch control – Sensitivity.

Introduction

The introduction of glycoprotein E (gE)-negative marker vaccines against Aujeszky's disease virus (ADV) made it possible to detect pigs infected with wild type ADV in vaccinated populations by using serological assays that detect antibodies to gE (3, 11, 12, 13). Since then, different ELISAs for the detection of gE-antibodies have been used in many control and eradication campaigns worldwide (9). In Belgium, the use of ADV-gE ELISAs is regulated by law, and until 2008 the diagnostic test kits of only one manufacturer were licensed for the eradication programme. To guarantee a high level of reliability of the ADV-gE ELISAs, the Belgian National Reference Laboratory for Aujeszky's disease (VAR) performs an elaborate control procedure on each ADV-gE ELISA batch before its release on the Belgian market. Not only are the analytical test

characteristics with regard to sensitivity and specificity (5) measured, but other characteristics such as 'ease of use' and precision (repeatability and reproducibility) are also tested (2) and compared with predefined minimum criteria. Only batches that fulfil these requirements are licensed.

One of the most important criteria during batch control is that the analytical sensitivity should be high enough to detect antibodies in the OIE reference serum ADV1 at a dilution of 1:8, as defined by the Decision of the European Commission (EC) (4).

In the present study, despite the use of licensed batches, some of the routine test runs were not validated because of lack of sufficient sensitivity. Although all technical parameters were controlled according to the quality management system (ISO 17025), the temperature for the incubation step at room temperature (RT) was measured according to the instructions of the manufacturer. However, RT is defined rather arbitrarily, because it may be any temperature between 18°C and 25°C or even higher, depending on the definition. As far as the authors are aware, no data have been reported regarding the influence of the incubation temperature on the analytical sensitivity of the test. The present study aimed to evaluate the role of the incubation temperature on the qualitative test results. The reference samples ADV1, diluted 1:8, and ADV gE Q (ADVQ) were used during 94 different runs using five different batches of a commercial ADV gE-ELISA.

Materials and methods

Aujeszky's disease virus gE enzyme-linked immunosorbent assay

Every batch of a commercial ADV gE-ELISA is evaluated by the VAR following a standard operating procedure accredited to ISO/IEC 17025 (7), and all technical parameters are registered during the assays. Briefly, ELISA plates are incubated with different test samples for 1 h at RT. Following a wash step, anti-ADV g1 immunoglobulins conjugated with horseradish peroxidase are added and incubated for 20 min at RT. After a final wash step, substrate is added and the plate is incubated at RT for 15 min. Subsequently, the reaction is stopped by the addition of a stop solution and the optical density (OD) is read at 650 nm using a microtitre plate reader.

Samples

For batch control purposes, the OIE/Community reference serum was used as a test sample. This reference serum was obtained from the OIE Reference Laboratory for Aujeszky's disease in France (www.oie.int/eng/OIE/organisation/en_LR.htm) and was reconstituted before use according to the accompanying data sheet instructions. The ADV1 is a serum sample obtained from a pig that was vaccinated twice against ADV and inoculated three times with the Kojnock ADV strain. The serum was collected 65 days after the first vaccination. Standardisation was carried out and a dilution of 1:180 was recommended for the reference serum ADV1. For routine use of the test kit, additional control sera, as well as the positive (PC) and negative control (NC) sera included in the kit, are tested in every run. These additional control sera consist of a positive serum, a negative serum and the ADV1 diluted 1:8 in porcine serum that is negative for antibodies specific to ADV gE. The positive internal serum was obtained from a pig infected experimentally with ADV. The positive status of the serum was confirmed by the serum neutralisation assay. The negative serum was obtained from a pig that originated from a certified ADV-negative farm, and the negative status of the sample was also confirmed by serum neutralisation assay. Aliquots of both control sera were lyophilised in vials and stored at 4°C. The ADV1 was provided by the OIE Reference Laboratory. The OD values and the corresponding standardised values (percentage inhibition) of all control sera were charted and monitored using the Multi QC program (3) (www.multiqc.com).

Each test run was validated when the validation criteria of the manufacturer were complied with and when all the additional control sera were validated following the Westgard rules (14, 15). Briefly, a test run was rejected if one of the control values exceeded the control limit of three standard deviations from its mean.

Data set

For this study, data were taken from 94 different runs in which the ADV1 OIE reference sample was used. The following variables with their corresponding codes are considered in this study:

– the percentage inhibition (% INH), the response variable of interest (continuous variable), is calculated according to the manufacturer's instructions:

> % INH = (mean OD_{NC} – OD_{sample})/mean OD_{NC} – mean OD_{PC}) × 100, where NC and PC stand for negative and positive kit control, respectively. A cut-off value of 40% INH is used to dichotomise the result as being positive (more than or equal to 40%) or negative (less than 40%)

– the incubation temperature (°C) of the ADV1 OIE reference sample, recorded during an ELISA run

– lot: the batch number used during the run. In this study, five different batches were used: 001KW (n = 18), 272BU (n = 17), 341GW (n = 20), 70EU (n = 17) and 819AW (n = 22)

– result: the status of the ADV1 OIE reference sample, which is a binary indicator for the final interpretation of the sample, coded as:

result = 0 for % INH <40% (sample is negative)

result = 1 for % INH ≥40% (sample is positive).

Methodology and statistical analysis

All data management and statistical analyses in this report were performed using SAS® System (SAS 9.1., SAS Institute Inc., 1999), whereas graphs (explorative data analysis) were obtained using the open source software R in the Epical package (http://cran.r-project.org). In order to describe the relationship between the incubation temperature and % INH, a first model was considered with % INH as the dependent variable and temperature as the predictor variable. The first linear model used all the data and was of the form:

$$Yi = \beta_0 + \beta_1 X_{i1} \quad [1]$$

Where Yi equals % INH, X_{i1} stands for the incubation temperature, and β_0 and β_1 are the parameters for the overall intercept and slope of the model, respectively.

A second model further considered the batch number of the ELISA kit as a qualitative predictor.

The following linear model was considered:

$$Yi = \beta_0 + \beta_1 X_{i1} + \beta_2 X_{i2} + \beta_3 X_{i3} + \beta_4 X_{i4} + \beta_5 X_{i5} + \varepsilon_i \quad [2]$$

Where

Yi = % INH

X_{i1} = incubation temperature

X_{i2} = 1 if batch number = 001KW, 0 otherwise

X_{i3} = 1 if batch number = 272BU, 0 otherwise

X_{i4} = 1 if batch number = 341GW, 0 otherwise

X_{i5} = 1 if batch number = 70EU, 0 otherwise

In this equation, β_0 is the intercept, or the Y value when χ = 0 (batch number 819AW was chosen as the reference). In addition, β_1 is the slope of the regression line (i.e. the change in % INH corresponding to a unit change in X_i or incubation temperature). The error terms are assumed to be normally distributed and to have common variance.

Results

In 20% of the test results (19/94 runs) the ADV1 OIE reference sample was negative (n = 9 for 70EU, n = 5 for 272BU, n = 3 for 341GW and n = 2 for 819AW). In all these cases, the incubation temperature was above 20°C. Table I summarises the % INH and incubation temperatures according to the different batches. From this table, it is apparent that the variance for both variables among the different batches is very similar, although a slightly higher variance was observed for batches 70EU and 272BU.

Table I
Summary of the variables incubation temperature (°C) and percentage inhibition (% INH) calculated according to the manufacturer's instructions

Batch number	Variable	Number of runs	Mean	Standard deviation	Min.	Max.
001KW	°C	18	25.278	1.487	22	27
	% INH	18	44.056	2.920	40	52
272BU	°C	17	22.706	1.714	20	27
	% INH	17	43.294	7.540	26	54
341GW	°C	20	22.850	1.368	20.5	24.5
	% INH	20	44.200	5.074	34	53
70EU	°C	17	24.206	1.047	22	26
	% INH	17	38.235	7.579	21	49
819AW	°C	22	23.545	1.281	21.5	26
	% INH	22	46.364	4.706	36	53

The data generated by model 1 (Fig. 1) demonstrate a significant effect of the incubation temperature on the % INH of the ADV1 reference sample ($p < 0.005$). Residual analyses showed that the data were normally distributed. The overall intercept (β_0) was 17.74 with a standard error (se) of 8.84, and the estimate for the slope (β_1) was 1.08 (se = 0.38 and 95% confidence interval [CI] ranging from 0.34 to 1.83). Consequently, the sample incubation temperature should be at least 22°C in order to have a positive result (% INH ≥40) for the ADV1 OIE reference sample.

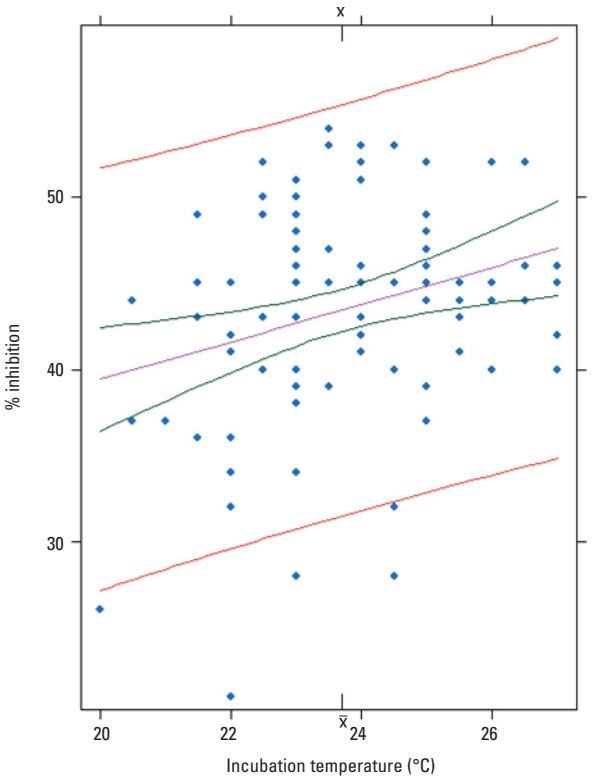

Fig. 1
Model 1: Influence of incubation temperature on the percentage inhibition of the ADV1 OIE reference sample
Blue dots are the observed responses (% inhibition). The green and red lines represent the 95% confidence limits and predicted limits, respectively

Table II shows the estimates for model 2. The analysis indicates that the use of different batches has a significant effect. Fixing the slope at 1.87 for each batch results in a positive test score for the ADV1 reference sample when the incubation temperature ranges from 20.15°C (batch 819AW) to 26.4°C (batch 70EU) (Fig. 2).

Table II
Estimates, standard errors (se) and p-values for model 2, showing the results for each batch

Parameter	Estimate	Standard error	p > |t|
Intercept	2.31	9.289	0.8038
Temperature (°C)	1.87	0.392	<0.0001
Batch 001KW	–5.55	1.769	0.0023
Batch 272BU	–1.50	1.692	0.3782
Batch 341GW	–0.86	1.611	0.5938
Batch 70EU	–9.36	1.680	<0.0001
Batch 819AW	(ref)		

Discussion

In the present study, the influence of the incubation temperature and the batch composition of a commercial ADV gE-ELISA was investigated in more detail. The results obtained using the OIE ADV1 reference sample demonstrated a significant effect of both the incubation temperature and the batch composition on the analytical sensitivity of the ELISA kit.

As far as the authors are aware, differences in sensitivity among different ADV gE-ELISA kits have been described (1, 6), but not those among different batches of the same kit. Arias *et al.* suggest that differences in analytical sensitivity between kits could be explained by the use of different specific anti-gI monoclonal antibodies (1). Given that in the present study the same kit was used, this explanation was not applicable. However, a considerable influence of the incubation temperature on the sensitivity of an ELISA was also observed by Sako *et al.* (10), who examined the influence of the incubation temperatures in each process of an ELISA for detection of watermelon mosaic and cucumber mosaic viruses. In their study different incubation temperatures were evaluated that fluctuated rather widely between 6°C and 37°C.

Twenty percent of the test runs performed in the current study resulted in a negative score for the ADV1 reference sample, even if the incubation temperature was higher than 20°C and thus also above the minimum of 18°C proposed by the manufacturer of the kit. As a consequence, the sensitivity of the test runs performed at 20°C was insufficient, according to the decision of the European Commission (4).

In the framework of the intra-Community trade in pigs and with respect to Aujeszky's disease, it is important that countries within the European Community use anti-ADV gE ELISA kits with the same sensitivity. Indeed, each Member State must be able to guarantee the negative status of animals that are exported to another Member State to avoid the possibility of spreading the virus. On the basis of the results obtained herein, it can be concluded that the use of the same reference sample by the different national reference laboratories during batch control is not enough to totally guarantee the sensitivity of this ELISA. Indeed, more stringent conditions should be defined to minimise fluctuations in the test results, and this analysis clearly shows that the test runs should be performed with an incubation temperature of at least 22°C (Fig. 1) and not 18°C as recommended in the instructions supplied by the manufacturer.

In addition to the incubation temperature, this study also demonstrated the significant influence of the batch composition on the analytical sensitivity of the ADV gE

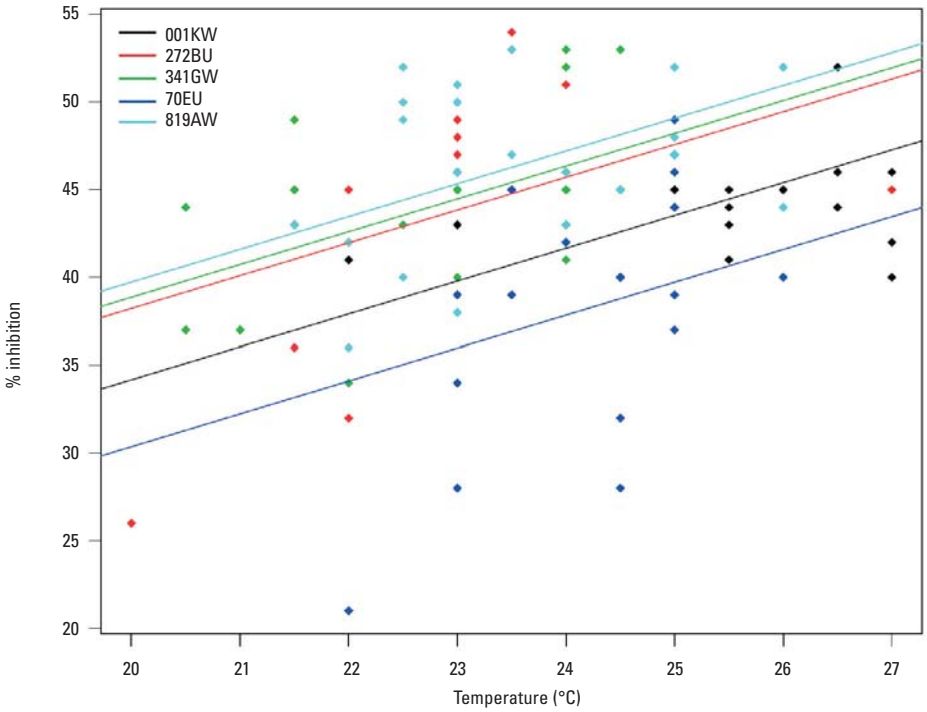

Fig. 2
Regression lines derived from model 2, showing the relationship between % inhibition and temperature for each batch

ELISA kit. For instance, an incubation temperature of 20.2°C was necessary to obtain a positive score for the ADV1 reference serum with batch 819AW, while for batch 70EU a higher incubation temperature of 26.4°C was required to achieve the same qualitative result for ADV1. The reasons for such differences among batches of the same kit might be sought at the level of one of the batch components or a combination of the components used to constitute the kit. For instance, the use of different batches of substrate or conjugate, and/or the coating process of the ELISA plates, could contribute, separately or as a whole, to the differences observed during this study. Indeed, the influence of different coating concentrations on the final reaction in an ELISA was also observed by Koenig (8). In this case, with a standardised commercial ELISA kit, it is unlikely that there will be great variations in coating concentrations, but it cannot be excluded that small variations in coating concentrations could have caused the differences observed in the current study. Although the exact influence of each of the components of the ELISA kit was not investigated, this study highlights the necessity to reflect on the importance of the batch and/or on the criteria used to define a batch.

The criteria for batch release of kits for the detection of specific anti-ADV gE antibodies are specified by the European Commission Decision (4). In view of greater harmonisation and standardisation of batch evaluation and release, however, the analysis presented here suggests that a well-defined incubation temperature is needed. In addition, it is recommended that this incubation temperature be evaluated for each batch separately. Indeed, the results show that a difference of only 0.5°C is sufficient to obtain a different qualitative result for the ADV1 reference sample between two batches (e.g. 819AW versus 341GW). Consequently, if the incubation temperature is not defined stringently for a certain batch, this could lead to a release in one country (laboratory) but a rejection in another country (laboratory) owing to a different qualitative result for the reference sample tested.

This study also demonstrates that the use of a weak positive primary reference standard, as recommended by Wright *et al.* (16), is not only a necessary tool to analyse the analytical sensitivity of each batch and to compare it among different batches of the same kit but also to ensure that the minimum required analytical sensitivity is obtained during each test run.

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Influence de la température d'incubation et de la composition des lots sur la sensibilité d'une épreuve immuno-enzymatique utilisée pour déceler la glycoprotéine E (gE) du virus de la maladie d'Aujeszky

A.B. Cay & Y. Van der Stede

Résumé

Les auteurs décrivent une situation où des séries de routine de l'épreuve immuno-enzymatique (ELISA) utilisée pour déceler la présence du virus de la maladie d'Aujeszky (ADV) n'ont pas pu être validées à l'issue du contrôle qualité, malgré le fait que les lots de réactifs ELISA utilisés avaient été dûment autorisés et que les tests avaient été réalisés conformément au système de contrôle de la qualité décrit par la norme ISO/IEC 17025, et ce même après vérification de tous les paramètres techniques. Deux paramètres ont néanmoins été suspectés d'avoir pu occasionner ces épreuves/séries non validées, à savoir les variations de la température d'incubation et la composition des lots. Une étude a donc été réalisée pour déterminer l'influence de la température d'incubation et de la composition des lots sur la sensibilité analytique de l'épreuve ELISA. Le sérum de référence ADV1 de l'Organisation mondiale de la santé animale (OIE) a été dilué au rapport 1:8 et testé sur 94 séries différentes d'épreuves ELISA visant à déceler la présence des anticorps dirigés contre la glycoprotéine E du virus de la maladie d'Aujeszky, en utilisant différents lots à différentes températures d'incubation. La température d'incubation et la composition des lots se sont révélées avoir une influence significative sur les résultats qualitatifs obtenus avec le sérum de référence de l'OIE. Au vu des résultats de cette étude, les auteurs recommandent une température d'incubation d'au moins 22 °C. L'étude n'a pas permis de déterminer quelles étaient les composantes des lots ayant pu entraîner les variations de sensibilité constatées.

Mots-clés

Contrôle de lots – Épreuve immuno-enzymatique pour la détection d'anticorps ADV-gE – Sensibilité diagnostique – Virus de la maladie d'Aujeszky.

Influencia de la temperatura de incubación y los componentes del lote sobre la sensibilidad de un ensayo inmunoenzimático para detectar la glucoproteína E (gE) del virus de la enfermedad de Aujeszky

A.B. Cay & Y. Van der Stede

Resumen

Pese a utilizar lotes homologados de un ensayo inmunoenzimático (ELISA) de detección del virus de la enfermedad de Aujeszky y a aplicar la técnica siguiendo un sistema de control de calidad conforme a la norma ISO/IEC 17025, algunas de las series sistemáticas de la prueba no resultaron válidas con arreglo a los criterios de calidad, aun cuando se mantuvieran controlados todos los parámetros técnicos. Se determinó que la incubación a distintas temperaturas y la composición de los lotes eran sendos parámetros que podían generar pruebas/series no válidas. A partir de ahí se estudió la influencia de ambos

parámetros sobre la sensibilidad analítica de la mencionada técnica ELISA. Tras llevarlo a una dilución de 1:8, el suero ADV1, que es el de referencia de la Organización Mundial de Sanidad Animal (OIE), fue sometido a 94 series del ELISA de detección de la glucoproteína E realizadas con distintos lotes y a diferentes temperaturas de incubación. Se observó que tanto la temperatura como los componentes del lote influían significativamente en el resultado cualitativo que deparaba el suero de referencia de la OIE. Atendiendo a los resultados del análisis, se recomendó una temperatura de incubación de al menos 22°C. No se estudió cuál de los componentes del lote daba origen a las diferencias de sensibilidad observadas.

Palabras clave
Control de lotes – Ensayo inmunoenzimático de detección de la glucoproteína E del virus de la enfermedad de Aujeszky – Sensibilidad – Virus de la enfermedad de Aujeszky.

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