Diagnostic performance of the pourquier ELISA for detection of antibodies against mycobacterium avium subspecies paratuberculosis in individual milk and bulk milk samples of dairy herds

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DIAGNOSTIC PERFORMANCE OF THE POURQUIER ELISA FOR DETECTION OF ANTIBODIES AGAINST MYCOBACTERIUM AVIUM SUBSPECIES PARATUBERCULOSIS IN INDIVIDUAL MILK AND BULK MILK SAMPLES OF DAIRY HERDS

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Running title: Performance of a commercial ELISA for detection of antibodies against M. avium subsp. paratuberculosis in individual and bulk milk samples of dairy cows

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Abstract

The objective of the study was to determine the diagnostic performance of the Pourquier ELISA for detection of antibodies against *Mycobacterium avium* subsp. *paratuberculosis* (Map) in individual milk samples and in bulk milk samples. For individual milk samples the specificity of the Pourquier ELISA was estimated by testing a panel of individual milk samples from certified Map-free herds. The relative sensitivity of the assay in individual milk samples and agreement of the results with those of serum samples was estimated by testing panels of paired serum-milk samples from seropositive cattle, whole-herd investigations, and moderate or heavy shedders.

The specificity of the ELISA for individual milk samples was still 99.8% at a cut-off of 20% sample to positive (S/P) value, clearly lower than the cut-off defined by the manufacturer (30% S/P). The relative sensitivity for individual milk samples as compared with positive serum samples was 87% for a cut-off of 20% S/P, and 80% for a cut-off of 30% S/P. The sensitivity of this ELISA for detection of high shedders was > 90% both for individual milk and serum samples, also agreement was very good (kappa = 0.91 for all paired samples).

The specificity of the Pourquier ELISA in bulk milk samples was investigated by testing bulk milk samples from certified Map-free herds. Feasibility of bulk milk testing was investigated by titrating ELISA positive individual milk samples in negative milk. In addition, 383 bulk milk samples from herds with a known within-herd seroprevalence were tested.

The specificity of the ELISA for bulk milk samples was 100% at a cut-off of 12.5% S/P. At the cut-off recommended by the manufacturer (30% S/P) performance of the bulk milk ELISA related to herd status (≥2 seropositive cows) was rather poor, corresponding with a sensitivity of 24% and a specificity of 99% relative to serology. However, at the revised cut-off for bulk milk of 12.5% S/P and a within-herd seroprevalence of ≥ 3%, sensitivity and specificity relative to serology were 85% and 96%, respectively. Given the current herd-level seroprevalence in the Netherlands, these test characteristics corresponded with positive and negative predictive values for bulk milk of 67% and 94%, respectively. In conclusion, the diagnostic performance of the Pourquier ELISA for individual
milk samples creates opportunities for a cheaper and more feasible testing scheme, while the diagnostic
performance for bulk milk samples warrants further consideration.

Keywords: M. avium subsp. paratuberculosis; ELISA; antibodies; diagnosis; serum; individual
milk; bulk milk; sensitivity; specificity; agreement; diagnostic performance; titration
1. Introduction

Paratuberculosis, caused by *Mycobacterium avium* subsp. *paratuberculosis* (Map), is a frequently present infectious disease in dairy cattle herds in many developed countries (Collins et al., 2005). In the Netherlands, as in other countries, much effort has been invested in the implementation of control programs, certification of herds and evaluation of these programs (Muskens et al., 2000; Groenendaal et al., 2003; Weber et al., 2004; Weber et al., 2006).

Detection of antibodies against Map by ELISA technology is an important tool in many regional and national control schemes for paratuberculosis. Although the relative sensitivity of ELISA as compared with faecal culture is rather low, especially for light shedders (van Schaik et al., 2003; Collins et al., 2005), ELISA technology has gained an important place in herd-based testing schemes because of its low cost and high-throughput potential. However, to obtain an acceptable herd sensitivity many cattle have to be tested, with sample size being negatively correlated with herd specificity. Therefore, in 1999 a large validation study with commercially available ELISAs was performed (van Maanen et al., *Mycobacterium paratuberculosis* Antibody detecting ELISAs, internal validation report Animal Health Service). Subsequently, an ELISA (Institut Pourquier, ELISA Paratuberculosis Antibody screening) was selected and implemented with a specificity of 99.8% and an overall relative sensitivity (as compared with faecal culture) of 40.8%. The test characteristics of the Pourquier ELISA also appeared to be quite satisfactory for small ruminants (Gumber et al., 2006).

Recently, a thorough evaluation of five ELISAs for diagnosis of bovine paratuberculosis was published. (Collins et al., 2005). In this study, the Pourquier ELISA also demonstrated an excellent specificity of ≥ 99.8%. The only milk ELISA described in this study, however, was not sold as a diagnostic kit but offered as a diagnostic service by a Michigan laboratory.

Adaptation of ELISA technology for milk samples for testing dairy cattle and herds would be very cost-effective for several reasons. In Denmark, much experience has been obtained with testing of individual milk samples in an in-house ELISA (Nielsen, 2002; Nielsen et al., 2002a; Nielsen et al., 2002b; Kudahl et al., 2004). However, only scarce literature is available about the feasibility of bulk
milk testing for diagnosis of bovine paratuberculosis (Nielsen et al., 2000).

According to the manufacturer the Pourquier ELISA can also be used for testing milk samples. The manufacturer, however, could only supply limited validation data. Therefore, in this study we evaluated the diagnostic performance and feasibility of testing individual milk samples and bulk milk samples in a commercially available ELISA (Institut Pourquier, ELISA Paratuberculosis Antibody screening) at optimised cut-off values.
2. Materials and Methods

2.1 Individual milk samples

Panel A consisted of individual milk samples (n=435), obtained from ten different certified Map-free herds (Groenendaal et al., 2003; Weber et al., 2004; Ezanno et al., 2005; Weber et al., 2006) with a 5 to 8 years history of negative annual whole-herd faecal culture and no introduction of cattle from other herds during this period to determine specificity and optimise the cut-off of the ELISA kit.

Panel B consisted of individual milk samples (n=52) from different seropositive cattle originating from infected herds to determine relative sensitivity of antibody detection in milk versus serum.

Panel C consisted of individual milk samples (n=30) from cattle tested seropositive in the previous 2-3 months in a study with twice yearly serological monitoring of a cohort of Map-infected dairy herds.

Panel D consisted of individual milk samples (n=300) from six different infected herds, samples were obtained cross-sectionally 1-3 months after the last serological investigation from all cattle in milk.

Panel E consisted of individual milk samples (n=36) from cattle recently diagnosed as moderate or heavy shedders either by faecal culture or by direct acid fast stain on faecal samples. Simultaneously also faecal samples and serum samples were taken to determine relative sensitivity of antibody detection in milk and serum for moderate and heavy shedders (resp. 10-100 or > 100 CFU on four Löwenstein-Jenssen slants after 8 weeks culture period, respectively, (Kalis et al., 2000). To prevent premature removal of faecal shedders from the herd, farmers were asked for their consent to take individual milk samples, faecal samples and blood samples by the local practitioner before reporting the faecal culture results.

2.2 Titrations of individual milk samples

To determine feasibility of pooling or bulk milk testing, the majority of ELISA positive individual milk samples (either from seropositive cattle, n=64, or from moderate and heavy shedders, n=32) were serially diluted in two-fold dilutions in negative milk from a certified Map-free herd and titres were calculated.
2.3 Bulk milk samples

Bulk milk samples (n=110) were obtained from 110 certified Map-free herds to determine specificity and optimise the cut-off.

In a randomized seroprevalence study in the Netherlands in 2004, bulk milk samples were obtained simultaneously with serum samples from 383 dairy herds (21,411 individual serum samples with a mean number of 53 sera per herd). Bulk milk samples were defatted by manual removal of the cream layer after storage overnight at 4-8 ºC and subsequently stored in 1 mL aliquots at -20 ºC. These bulk milk samples (n=383) were tested to determine the relationship between bulk milk ELISA result and within-herd seroprevalence.

2.4 Between-test variability

A subset of 64 ELISA positive individual milk samples were retested with a week interval and the correlation coefficient between results of both tests was determined. These sera were randomly selected, and represented the whole range of low-positive to high positive results (Fig. 4).

2.5 Absorbed ELISA

All samples were tested in a commercially available ELISA kit (Institut Pourquier, ELISA Paratuberculosis Antibody screening) according to the instructions of the manufacturer. As a first step in the test protocol serum samples were diluted to 1/20 and milk samples to 1/2 in dilution buffer containing Mycobacterium phlei extract, also according to the instructions of the manufacturer. Results were expressed as percentage S/P, calculated by 100 x (OD value of the sample - the OD value of the negative control)/(OD value of the positive control - the OD value of the negative control). Cut-offs as recommended by the manufacturer are for serum samples < 60% S/P negative, 60-70% S/P ambiguous, and >70% S/P positive, and for milk samples < 30% S/P negative, 30-40% S/P ambiguous, and >40% S/P positive.
2.6 Statistical analysis

Test agreement, sensitivity, specificity and 95% confidence intervals were calculated using WinEpiscope 2.0 (N. de Blas, C. Ortega, K. Frankena, J. Noordhuizen, M. Thrusfield: http://www.clive.ed.ac.uk/wineписьcope/). Scatter plots and correlation analyses were performed using Microsoft EXCEL 2000. ROC analysis was performed in SPSS 10.0.
3. Results

3.1 Specificity of ELISA for individual milk samples

To determine the specificity of the milk ELISA the milk samples indicated as panel A (n=435) were used. Fig. 1 shows the frequency distribution and the cumulative distribution for S/P values of individual milk samples. Using the cut-off of 30% S/P value as defined by the manufacturer the specificity was 100%. To achieve a similar specificity for individual milk samples as achieved for serum samples, a cut-off of 20% S/P was selected, yielding a specificity of 99.8% (95% CI: 99.3-100%) in the individual milk sample set investigated.

3.2 Relative sensitivity and agreement of ELISA for individual milk samples as compared with serology

To determine the relative sensitivity of the milk ELISA in relation to serology the milk samples of panel B and C (n=82) were used. At a cut-off of 20% S/P for individual milk and a cut-off of 60% S/P – as defined by the manufacturer - for serum, 71 out of 82 seropositive cattle scored positive in individual milk samples (relative sensitivity of 87% (95% CI: 79-94%)). At a cut-off of 30% S/P and 60% S/P for individual milk and serum samples, respectively (both as defined by the manufacturer) the relative sensitivity for individual milk samples was 80% (95% CI: 72-89%). When for serum samples a cut-off of 90% S/P was used – previously defined by the manufacturer and still used in our laboratory for specificity considerations – 71 out of 74 seropositive cattle scored positive in individual milk samples at a cut-off of 20% S/P (relative sensitivity of 96% (95% CI: 92-100%)).

Agreement beyond chance, expressed by kappa-values between individual milk results on the one hand and serum results on the other hand was high with a kappa-value of 0.91 (95% CI: 0.87-0.95) when all samples of panels B-E were included (n=417). Also, in panel D, a cross-sectional sample (n=300) in six different infected herdsof milk samples 1-3 months after the serological investigations the agreement between serum and individual milk results was high (kappa=0.83 (95% CI: 0.72-0.94)). Furthermore, S/P values of serum samples and individual milk samples were clearly correlated
(R=0.92) as demonstrated in Fig. 2, although not in a strictly linear way and with generally lower S/P values in milk than in serum.

3.3 Relative sensitivity of ELISA for individual milk and serum samples as compared with faecal shedding

To determine the relative sensitivity of the milk ELISA in relation to fecal shedding the samples indicated as panel E (n=36) were used. The agreement between ELISA results of individual milk and serum samples was very good with a relative sensitivity as compared to fecal shedding of 89% (95% CI: 79-99%). When only high shedders were included (based on the previous and/or current culture results, n=30) the relative sensitivity of this ELISA was 97% (90-100%) for both individual milk and serum samples.

3.4 Titre distribution of individual ELISA positive milk samples

In total, 97 ELISA positive individual milk samples from panel B-E were titrated in negative milk. The relationship between S/P value of undiluted milk samples and the log_{10} titres of the same samples is presented in Fig. 3. S/P values and titres were clearly correlated (R=0.90).

S/P values were arbitrarily categorized in four S/P classes and mean log_{10} titres were calculated (Table 1). The overall geometric mean titre of all ELISA positive individual milk samples investigated was 1.0 log_{10} or 1:10. For a category of moderate and high shedders the geometric mean titre of ELISA positive individual milk samples was 1.36 log_{10} or 1:23.

3.5 Between-test variability of ELISA results for individual milk samples

To get an impression of the within-laboratory reproducibility of the Pourquier ELISA for milk samples a set of ELISA positive individual milk samples from panel B-E (n=64) was tested twice with eight days interval in the same laboratory. Results are presented in Fig. 4. All samples scored positive again in the second test and S/P values correlated very well between the two tests with correlation and
regression coefficients close to 1 (r = 0.97; regression coefficient = 1.06).

3.6 Specificity of ELISA for bulk milk samples

Fig. 5 shows the frequency distribution and the cumulative distribution for S/P values of bulk milk samples (certified Map-free herds, a subset of the herds mentioned in paragraph 2.3, n=110). Using the cut-off of 30% S/P value as defined by the manufacturer the specificity was 100%. A cut-off of 12.5% S/P, however, still yielded a specificity of 100% in the bulk milk sample set investigated.

3.7 Diagnostic performance of ELISA for bulk milk samples related to within-herd seroprevalence

Bulk milk ELISA results were initially interpreted as described by the manufacturer for individual milk samples (< 30% S/P negative, ≥ 30% S/P suspect/positive). Diagnostic performance of the Pourquier ELISA for bulk milk samples related to within-herd seroprevalence is summarised in Table 2. From the 383 herds participating in a randomized seroprevalence study with a bulk milk sample, 267 herds were completely seronegative, 62 herds had one seropositive and/or suspect animal, and 54 herds had two or more seropositive and/or suspect animals. There was a fair correlation between S/P values of bulk milk samples and within-herd seroprevalence (r = 0.70). Values for sensitivity, specificity and overall diagnostic potential as indicated by the Area Under the Curve (AUC) are presented in Table 2 for different herd status criteria based on the interpretation of test results in practise (absolute # of positives) and for different prevalences (≥2% to ≥5%). Test characteristics are presented at a cut-off of 30% S/P as indicated by the manufacturer and at a cut-off of 12.5% S/P. The latter cut-off was chosen because it corresponded with the cut-off resulting in 100% specificity for bulk milk samples for certified Map-free herds, and this cut-off also yielded a high specificity for the bulk milk samples from the seronegative herds in the seroprevalence study.

Because bulk milk samples originated from a randomised prevalence study in the Netherlands, also positive and negative predictive values (PVP and PVN) could be calculated. For example, for a herd with ≥3% seroprevalence which is the most common seroprevalence in the Netherlands (Muskens et
al., 2000) and a cut-off for bulk milk of 12.5% S/P, sensitivity and specificity were 85% and 96%, respectively, and the PVP and PVN were 67% and 94%, respectively. Experiences with the Dutch paratuberculosis programmes since 1998 demonstrated that a level of $\geq 3\%$ seroprevalence (all present cattle $\geq 3$ years of age tested) also indicate an infection level of the herd where one or more heavy shedders are present. It should be noted that in almost all herds (12 out of 14) that had a prevalence $<3\%$ and tested positive in bulk milk one or more seropositive animals were detected.
4. Discussion

One of the advantages of many years of paratuberculosis research and control in the Netherlands is the presence of a large pool of certified Map-free herds with a long history of negative ELISA and faecal culture test results. From these herds individual milk samples and bulk milk samples were taken and investigated in the Pourquier ELISA. As shown in Figs. 1 and 5, cut-offs corresponding with a specificity of nearly 100% for both individual and bulk milk samples could be (much) lower than the cut-off of 30% S/P defined by the manufacturer. For individual milk samples the revised cut-off was higher (20% S/P) than for bulk milk samples (12.5% S/P), but much lower than for serum samples (60% S/P). Apparently, un-specific reactions are more predominant in serum samples than in milk samples, allowing lower cut-offs and lower pre-dilutions for milk samples, as described as well for several other infectious diseases (Bjorkman et al., 1997; Kramps et al., 1999; Beaudou et al., 2001; Nielsen et al., 2002a; Bartels et al., 2005; Schares et al., 2005).

The relative sensitivity of testing individual milk samples (at revised cut-off) as compared with serum samples was high, and varied between 87 and 96%, depending on the cut-off used for serum samples. In recent years, the manufacturer has decreased the cut-off for serum samples to 60% S/P, but because of concerns of losing specificity we maintained the formerly prescribed cut-off of 90% S/P. The agreement beyond chance, as expressed by kappa-values, between individual milk and serum results was high, also for the unbiased cross-sectional sample panel D.

High relative sensitivities for individual milk samples and good agreement with serum results for diagnosis of bovine paratuberculosis have also been described by others (Sweeney et al., 1994; Winterhoff et al., 2002; Nielsen et al., 2002a; Collins et al., 2005).

On the other hand, several authors have reported poor sensitivities and/or poor agreements and correlations for individual milk relative to serum (Hardin and Thorne, 1996; Hendrick et al., 2005a; Hendrick et al., 2005b). Several factors may play a role, such as the different commercial or in-house ELISAs that were evaluated in the publications. Moreover, the stage of lactation in which the paired samples were taken may have played a role. (Nielsen et al., 2002a) demonstrated that in the beginning...
of the lactation the probability of being positive was highest in the milk ELISA, while in the serum ELISA the probability of being positive was highest at the end of lactation. Because of practical considerations for implementation of individual milk testing into our control program we did not take the stage of lactation into account.

We also evaluated the diagnostic performance of individual milk and serum samples for moderate to heavy shedders. Heavy shedders in particular play an important role in the within-herd transmission of paratuberculosis, with moderate shedding as a preceding transitional stage (van Schaik et al., 2003; Collins et al., 2005). For a set of 36 predominantly high shedders, the relative sensitivity of the Pourquier ELISA using faecal culture as a gold standard was 89% both for serum and for milk. Sensitivities increased to 97% when the moderate shedders were excluded. High sensitivities of ELISAs for detection of heavy shedders were also reported by others (van Schaik et al., 2003; Collins et al., 2005).

Contrary to some other authors (Hardin and Thorne, 1996; Hendrick et al., 2005b) we found a clear, although not linear, correlation between result of individual milk and serum samples (Fig. 2). However, S/P values in individual milk samples where generally lower than in serum samples, as reported by others (Winterhoff et al., 2002). Also the reproducibility of S/P values of positive milk samples appeared to be good, which is important for consistent results in certification schemes, control programs and longitudinal studies.

Because bulk milk is essentially a pooled sample of individual cows, we determined the titre distribution of a panel of ELISA positive individual milk samples. Titres varied between 1/1 and 1/1024 with a clear correlation between the S/P value of the undiluted sample and the titre. Although this phenomenon matched our expectations, titres were in general rather low with a geometric mean titre of 1/10 of a milk panel obtained from seropositive cattle, and a geometric mean titre of 1/23 for a milk panel derived from moderate to high shedders. These results matched those of Arrigoni et al. (Institut Pourquier, personal communication) and would imply restricted possibilities for pooling of samples. In other words: for detection of infected herds (with a pooled milk sample) in general a 10%
seroprevalence would be the mean limit of detection and for detection of infected herds that contain at least one moderate to heavy shedders, a 4-5% seroprevalence would be the mean limit of detection. Subsequently, we analysed bulk milk samples of a large number of herds with known seroprevalence. Although many examples exist for bulk milk testing for other diseases, for paratuberculosis hardly any literature is available (Nielsen et al., 2000; Beyerbach et al., 2004). Nielsen et al. (2000) concluded that the technical performance of the ELISA was not sufficient to provide a tool for surveillance. Beyerbach et al. (2004) used a modification of a non-absorbed LAM-ELISA for bulk milk testing, and related the test results to within-herd test prevalence for individual milk samples. However, only 28 herds were involved in the study, and in our validation study and that of others (Collins et al., 2005) the LAM-ELISA lacked specificity, at least for serum samples.

Indeed, diagnostic performance of the bulk milk ELISA was rather poor at a low prevalence (≥1 seropositives in a herd; ≥2% seroprevalence in a herd) as demonstrated by low sensitivities, even at a much lower cut-off than defined by the manufacturer, and rather low AUC values in a ROC analysis. However, at prevalence levels of ≥3% and with the revised cut-off the bulk milk ELISA appeared to have diagnostic potential with a sensitivity and specificity of 85 and 96%, respectively. This would imply a detection level of 1 seropositive out of 30 cattle or 3 out of 100 cattle. (Beyerbach et al., 2004) reported for their bulk milk ELISA a sensitivity and specificity of 75 and 84%, respectively, at a within-herd prevalence level of 5%. The even lower cut-off for bulk milk samples may have contributed to more favourable results than we expected from the titration experiments. When bulk milk testing would be used as a first screening test for regional or national programs the negative predictive value would be particularly important. At a 3% seroprevalence level in test-positive herds, the negative predictive value of bulk milk would be 94%, which seemed quite acceptable. The positive predictive value was only 67% using the ≥3% seroprevalence criterium. On the other hand, in almost all “false-positive” herds one or more seropositive animals were detected.

In conclusion, the Pourquier ELISA can be used for testing individual milk samples as an alternative for individual serum samples, and currently individual milk samples are already routinely submitted to
our laboratory. Bulk milk testing warrants further consideration, and needs further evaluation.

Acknowledgements

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Table 1. Relationship between S/P values of ELISA positive undiluted individual milk samples and end-point dilutions in negative milk, presented as geometric mean titres per S/P% class, range of positive S/P values divided into four classes.

<table>
<thead>
<tr>
<th>Class S/P%</th>
<th>n</th>
<th>Mean log_{10} titre</th>
<th>Mean titre</th>
</tr>
</thead>
<tbody>
<tr>
<td>20-90</td>
<td>30</td>
<td>0.39</td>
<td>2</td>
</tr>
<tr>
<td>90-160</td>
<td>29</td>
<td>0.92</td>
<td>8</td>
</tr>
<tr>
<td>160-230</td>
<td>27</td>
<td>1.46</td>
<td>29</td>
</tr>
<tr>
<td>230-300</td>
<td>11</td>
<td>1.94</td>
<td>87</td>
</tr>
<tr>
<td>All classes</td>
<td>97</td>
<td>1.00</td>
<td>10</td>
</tr>
</tbody>
</table>
Table 2. Sensitivity and specificity of the Pourquier ELISA for bulk milk samples (n=383, n=267 bulk milk samples from seronegative herds) at two different cut-offs and six different herd criteria for seroprevalence (between brackets the number of herds fulfilling the criterion). Per herd criterion the AUC is given as an indicator of diagnostic potential of the test.

<table>
<thead>
<tr>
<th>Herd criterion</th>
<th>Cut-off 30% S/P</th>
<th>Cut-off 12.5% S/P</th>
<th>AUC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seropositive cattle</td>
<td>Se (%)</td>
<td>Sp (%)</td>
<td>Se (%)</td>
</tr>
<tr>
<td>=1 (62)</td>
<td>14</td>
<td>100</td>
<td>35</td>
</tr>
<tr>
<td>≥2 (54)</td>
<td>24</td>
<td>99</td>
<td>52</td>
</tr>
<tr>
<td>≥2% (82)</td>
<td>17</td>
<td>99</td>
<td>39</td>
</tr>
<tr>
<td>≥3% (50)</td>
<td>28</td>
<td>99</td>
<td>85</td>
</tr>
<tr>
<td>≥4% (32)</td>
<td>38</td>
<td>99</td>
<td>69</td>
</tr>
<tr>
<td>≥5% (22)</td>
<td>50</td>
<td>99</td>
<td>72</td>
</tr>
</tbody>
</table>
LEGENDS TO FIGURES

Fig. 1. Frequency distribution and cumulative distribution of ELISA results for individual milk samples from Map-certified herds.

Fig. 2. Relationship between ELISA results of paired individual milk and serum samples, dotted lines represent test cut-offs for individual milk and serum samples.

Fig. 3. Relationship between ELISA results (S/P%) of undiluted individual milk samples and log₁₀ titres of the same samples diluted in milk from a Map-certified herd.

Fig. 4. Between-test variability for individual milk samples in the Pourquier ELISA tested with an 8-day interval.

Fig. 5. Frequency distribution and cumulative distribution of ELISA results for bulk milk samples from Map-certified herds.
$y = 0.003x^2 - 0.109x + 10.7$

$R = 0.92$
Fig. 3

\[ y = 0.77 \ln(x) - 2.76 \]

\[ R = 0.90 \]

S/P values undiluted individual milk samples

Log10 titre individual milk samples

S/P values undiluted individual milk samples
Fig. 4

$y = 1.06x$

$R = 0.97$

S/P values individual milk samples D0

S/P values individual milk samples D8
Fig. 5

![Graph showing the distribution of S/P classes with cumulative frequency.](image)