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Filter paper is a simple and cost-effective transport medium for serological diagnosis of Peste des petits ruminants

Emeli Torsson[1*], Muhammad Abubakar[2], Karin Alvåsen[3], Shumaila Manzoor[2,] Nils Roos[1], Tebogo Kgotlele[4], Aamer Bin Zahur[2], Gerald Misinzo[4], Mikael Berg[1], Jonas Johansson Wensman[3]

[1] Swedish University of Agricultural Sciences, Department of Biomedical Sciences & Veterinary Public Health, P.O. Box 7028, SE-750 07 Uppsala, Sweden

[2] National Veterinary Laboratory, Park Road, Islamabad, Pakistan

[3] Swedish University of Agricultural Sciences, Department of Clinical Sciences, P.O. Box 7054, SE-750 07 Uppsala, Sweden

[4] SACIDS Africa Centre of Excellence for Infectious Diseases of Humans and Animals in Eastern and Southern Africa, College of Veterinary Medicine and Biomedical Sciences, Sokoine University of Agriculture, P.O. Box 3297, Chuo Kikuu, Morogoro, Tanzania

*corresponding author

Contact information: e-mail: emeli.torsson@slu.se, phone +4618-671704

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Conflict of interest

The authors declare that they have no conflict of interest.

Abstract

Peste des petits ruminants (PPR) is a highly contagious disease caused by peste-des-petits-ruminants virus. Following the successful eradication of the related rinderpest virus, a program to control and eradicate PPR was launched by the FAO and OIE. PPR is today present in many tropical countries where maintaining the cold chain for sample transportation is one of the major barriers for timely processing. Transport of samples on filter paper is a simple and cost-effective method, however validation and optimization is required to fully adapt this approach. The objective of this study was to evaluate and validate the use of filter paper in serological diagnosis of PPR. Blood samples (serum and filter paper) were collected from sheep and goats in both Tanzania and Pakistan and analysed using a PPRV-specific cELISA. The positive proportion was 10.7 % in Tanzania and 80 % in Pakistan when performing the analysis on serum. These results were then considered as reference and compared to the results from the filter papers analysed by the same cELISA. According to the statistical analysis the cut-off for a positive results for samples stored on filter paper was adjusted from < 50 % competition percentage to < 84 % in Tanzania and to < 69 % in Pakistan.

These results demonstrate that filter papers are an acceptable and cost-effective transport method of whole blood samples for later use in serological analysis.

Key words

Peste des petits ruminants, serological diagnosis, filter papers, transport, Tanzania, Pakistan

Introduction

Peste des petits ruminants (PPR) is a highly contagious and deadly disease caused by the peste-des-petits-ruminants virus (PPRV) (Gibbs et al., 1979, ICTV, 2016). The main hosts are sheep and goats, however disease in wild small ruminants and camels has also been reported (Abubakar et al., 2011, Aguilar et al., 2018, Mahapatra et al., 2015, Khalafalla et al., 2010). Clinical signs related to PPR include a high fever, ocular and nasal discharge, necrotic lesions in mucous membranes, pneumonia, diarrhoea, immunosuppression, and a high case fatality rate (up to 90%) in an epidemic setting (Torsson et al., 2016). Following the successful eradication of a related morbillivirus, rinderpest virus (Roeder et

56 al., 2013, Gibbs et al., 1979), the Food and Agricultural Organization of the United Nations (FAO) and
57 the World Animal Health Organization (OIE) have launched a program to control and eradicate PPR
58 (FAO, 2015).
59 Our understanding on the PPRV epidemiology has increased significantly in the last
60 years, however continued monitoring and strict biosecurity measures would underline the success of the
61 eradication program. Peste-des-petits-ruminants virus is currently present in north, central, east, and
62 west Africa, the Middle East and parts of Asia (OIE, 2016). Many of these are tropical countries where
63 maintaining the cold chain for sample transportation is one of the major barriers for timely processing
64 of samples (FAO, 2013). Filter paper as a mode of sample collection and transport has been suggested
65 previously (Michaud et al., 2007, Matheus et al., 2015, Randriamparany et al., 2016). For PPR, filter
66 paper has been proposed for long-term storage followed by detection and genotyping using PCR
67 (Michaud et al., 2007), but has not yet been studied in serological diagnosis. Transport of samples on
68 filter paper is a simple and cost-effective method, however more robust validation and optimization is
69 required to fully adapt this simple and cost-effective approach (Hopkins et al., 1998). The objective of
70 this study was to evaluate the use of filter paper, in comparison to serum, in serological diagnosis of
71 PPR using the OIE suggested competitive enzyme linked immunosorbent assay (cELISA) (Libeau et
72 al., 1995). We use two different kinds of filter paper, the Nobuto Filter Strips and, an even less expensive
73 alternative, chromatography paper. The filter papers were optimized and validated on clinical samples.
74

75 **Materials and methods**

76 ***Sample collection***

77 *Tanzania*

78 Sample collection was performed during June-July in 2015 in two districts (Kilombero and
79 Ulanga) in the Morogoro region (Torsson et al., 2017). This region was selected due to previous reports
80 of presence of PPR (Kgotlele et al., 2014, Misinzo et al., 2015). Ethical approval was received from the
81 Research Animal Council at the Swedish University of Agricultural Sciences (SLU ua 2017.1.1.1-1881).
82 Blood samples were collected from domestic sheep and goats, between the ages 3-12 month, from herds
83 of different sizes. Each animal was sampled in triplicate: serum in collection tubes using a vacutainer

84 system (BD Biosciences), and two different types of filter paper: Nobuto Filter Strips (NFS) (Advantec,
85 Dublin, CA, United States) and a chromatography paper (CP) (grade FN 100, weight 195 g/m^2, thickness
86 0.35 mm, capillary rise 115) (Sartorius AG, Goettingen, Germany). The CP was delivered in sheets
87 (260*410 mm) and prior to sample collection the sheet was cut into pieces similar in size to the Nobuto
88 Filter strips, approximately 10*50 mm.

89 Blood was collected from the jugular vein using sterile needles and vacutainer tubes without
90 additives (BD vacutainer, Plymouth, UK). Tubes were then opened and the two different filter papers
91 were inserted and allowed to soak in whole blood until about half of the paper was saturated with blood.
92 Filter papers were left to dry in a standing position, away from direct sunlight and at ambient temperature
93 (at sampling this was around 29-35°C). When dry, filter papers were stored separately, wrapped in a
94 paper envelope, at room temperature (20-28°C). Whole blood was left to coagulate and separate in a
95 vertical position in a cool box. After separation, the serum was transferred to cryotubes and stored at -
96 45°C until analysis.

97 *Pakistan*

98 Sample collection was performed during October-November in 2015 in the Punjab province.
99 Blood was collected from goats from the jugular vein using sterile needles and vacutainer tubes without
100 additives (BD vacutainer, Plymouth, UK). In Pakistan only the Nobuto filter strips were used for sample
101 collection. Filter strips were inserted into the vacutainer tube and the narrow part of the strip was allowed
102 to saturate with whole blood. Filter strips were dried in a standing position in room temperature away
103 from direct sunlight. When dry, the filter papers were labelled and sent to National Veterinary
104 Laboratory (NVL), Islamabad, via post. The corresponding serum samples were transported to NVL on
105 ice.

106 ***Preparation of filter papers and serological analysis***

107 All samples were analysed with *ID screen PPR competition ELISA* (sensitivity 94.5 %,
108 specificity 99.4 %; ID.Vet, Grabels, France), which is based on the recombinant nucleoprotein of PPRV
109 (Libeau et al., 1995). Serum was analysed according to manufacturer's instructions. Briefly the analysis
110 was performed as following: to a 96-well plate, pre-coated with recombinant PPRV nucleoprotein, 25µl
111 of sample was added and incubated for 45 minutes at 37°C. The plate was then washed 3 times before

112 adding the conjugate (100µl/well), followed by 30 minutes of incubation at 21°C. The wash was
113 repeated and 100µl/well of substrate solution was added and incubated for 15 minutes at room
114 temperature. As a last step, 100µl/well of stop solution was added to stop the reaction. Plates were read
115 at 450 nm.

116 The dried filter papers were prepared as following before use in the cELISA: From each filter
117 paper a piece of approximately 75 mm^2 was cut. This piece was then cut into 5-8 smaller pieces and
118 added to 150 µl of ELISA dilution buffer and incubated for 1 hour in room temperature. After
119 incubation, 50 µl of this solution was used in each well, which was otherwise performed in the same
120 way as the serum samples.

121 ***Statistical analysis***

122 The results from the serological assays performed on the filter papers were compared to the
123 assays performed on serum, which was considered as the gold standard. A few serological results were
124 between competition percentages 50-60, which according to the manufacturer are considered as doubtful
125 results. These results were considered as negative in the statistical analyses. All statistical analyses were
126 done in the statistical software *R,* version 3.2.2. (R Core Team, 2015). Cohen's Kappa was used to
127 calculate agreement between serum and filter paper assays. Agreement of results was further studied
128 using Bland-Altman plots, with the results from analysis on serum samples considered as the reference.
129 The dots in the Bland-Altman plot represents the difference between the measurements (serum vs. the
130 filter paper of each individual sample) on the y-axis and the average of the measurements on the x-axis
131 (Bland and Altman, 1999). In other words, the difference of the measurements is plotted against the
132 mean of the measurements and the coloured lines represents the mean difference. If the two tests were
133 to give the same results, all the dots would be centred around 0 on the y-axis (marked by the black
134 horizontal line) and the coloured line would overlap with the black line. Plots were produced using the
135 *blandr*-package (Datta, 2017). Receiver operating characteristic curve (ROC-curve) and Precision-
136 Recall curve (PRC) was produced by the *precrec*-package (Saito and Rehmsmeier, 2017). The *pROC*-
137 package was used to find the adjusted cut-off, including sensitivity and specificity, for filter paper assays
138 (Robin et al., 2011). Sensitivity and specificity were given the same weight when evaluating the adjusted
139 cut-off value.

140

141 **Results**

142 In Tanzania, 32 sheep and 164 goats were sampled, and in Pakistan, 60 goats were sampled.
143 Of the 196 animals sampled in Tanzania, 21 animals (10.7 %) were seropositive for PPRV antibodies
144 according to the ELISA on serum samples. When performing the ELISA using whole blood stored on
145 either NBS or CP, 11 samples (5.6 %) were positive when using the suggested < 50 % cut-off (Table
146 1A and 1B). In Pakistan, 48 animals (80 %) were seropositive according to assay on serum samples,
147 and 40 animals (66.7 %) were positive on assay on NFS (Table 1C).

148 The Bland-Altman plots indicate a systematic difference between the methods that is
149 comparable all over the test result interval (Figure 1A-C). To find an adjusted cut-off for the analysis
150 on the different kinds of filter papers a ROC-curve analysis was used (Figure 2 and 3). The ROC-curve
151 for the NFS in Tanzania had an area under the curve (AUC) of 0.988, where an AUC of 1 indicates a
152 perfect fit. The adjusted cut-off value for a positive sample was calculated to be < 84.6 % (instead of <
153 50 %) (Figure 2A), which would give a sensitivity of 95.2 % (95 % CI 85.7;100) and a specificity of
154 97.2 % (95 % CI 94.4;99.4) for the NFS saturated with whole blood. Cohen's kappa for the NFS first
155 using the <50 % cut-off was 0.66 (0.47-0.85). When adjusting the cut-off to 84.6 %, Cohen's kappa was
156 improved to 0.85 (0.74-0.97) (Table 2). The ROC-curve for the CP had an AUC of 0.983 and an adjusted
157 cut-off of < 84.3 %, sensitivity of 90.5 % (95 % CI 76.2;100), and specificity of 99.4 % (95 % CI
158 98.3;100) (Figure 2A). Cohen's kappa for the cut-off < 50 % was 0.62 (0.42-0.82) and on the adjusted
159 cut-off (< 84.3 %) was improved to 0.92 (0.83 – 1) (Table 2). Due to the data being unbalanced (with a
160 higher ratio of negative results compared to positive results) the performance of the filter papers were
161 further evaluated using a PRC (Figure 2B). The PRC gives the precision (equal to the positive predictive
162 value, PPV) for all values of recall (equal to the sensitivity of the test). A perfect test would have a
163 precision and recall of 1 and form a 90° angle in the right upper corner of the graph and an AUC of 1.
164 Our test shows an excellent performance level both on NFS (AUC 0.938) and CP (AUC 0.925) (Saito
165 and Rehmsmeier, 2015).

166 The results from the samples from Pakistan were analysed accordingly. The ROC-curve had
167 an AUC of 0.996 and a new cut-off of < 69.0 % (Figure 3A). This new cut-off had a sensitivity of 97.9

168 % (95 % CI 93.8;100) and a specificity of 100 % (95 % CI 100;100). The un-adjusted Cohen's kappa
169 was calculated to be 0.77 (0.58-0.96), and with the adjusted cut-off (< 69 %) improved to 0.95 (0.85-1)
170 (Table 3). The results in Pakistan were also unbalanced, however with a higher ratio of positive animals
171 compared to negative results, giving both very high precision and recall in the PRC (AUC 0.999) (Figure
172 3B).
173
174 **Discussion**
175 Since it was first described in 1942 (Gargadennec and Lalanne, 1942), Peste des petits
176 ruminants (PPR) has spread to most of Africa and Asia. These areas hold the majority of the world's
177 sheep and goat population, with an estimated 1.7 billion animals at risk (FAO, 2015). One of the major
178 obstacles for the control and eradication of PPR is the transport of samples from remote areas to
179 laboratory facilities for testing. Especially the cold chain can be difficult to maintain, which is important
180 to preserve the sample and prevent the proteins to degrade. Here we used filter papers as a highly cost-
181 effective method for transport of whole blood samples. Furthermore we have optimized the use of these
182 papers in a cELISA for serological diagnosis of PPR (Libeau et al., 1995). The filter papers performed
183 well in the analysis when compared to the results from corresponding serum samples tested in the same
184 cELISA. The AUC of the ROC curve was > 0.98 in all three examples (Figure 2A and 3A), which is
185 considered as an excellent accuracy of the test (Fawcett, 2006). ROC curves can however be misleading
186 when used on unbalanced data (data with a higher proportion of either negative or positive results), and
187 PRC was therefore produced as a complement (Figure 2B and 3B). In a PRC it is possible to see the
188 precision (equal to the positive predictive value, PPV) for all values of recall (equal to the sensitivity of
189 the test). The sensitivity values are plotted along the x-axis and the PPV along the y-axis. A perfect test
190 would have a precision and recall of 1 and form a 90° angle in the right upper corner of the graph, and
191 an AUC of 1, our three tests scored an AUC between 0.925 – 0.999. Additionally, Cohen's kappa was
192 used to measure the agreement between results from analysis performed on serum and analysis
193 performed on filter papers. Before adjusting the cut-off, Cohen's kappa for NFS was 0.66 in Tanzania,
194 0.77 in Pakistan, and for CP in Tanzania it was 0.62. These values are considered as a moderate to
195 substantial agreement (McHugh, 2012). When adjusting the cut-off and re-calculating Cohen's kappa

196 values they were raised to 0.85, 0.95, and 0.92, respectively, which are considered a near perfect
197 agreement (a value of 1 is seen as a perfect agreement) (McHugh, 2012). This demonstrates how
198 important it is to adjust the cut-off when using filter papers instead of serum in the analysis.

199 Sensitivity and specificity were over 90 % in all examples when the cut-off value for a positive
200 result was adjusted. The NFS gave a higher sensitivity and specificity in both countries (Tanzania: 95.2
201 % and 97.2 %, Pakistan: 97.9 % and 100 %) compared to the CP used in Tanzania (90.5 % and 99.4 %).
202 The NFS have been optimized for transport and storage of clinical samples and is expected to perform
203 better, however the CP is much cheaper (around 30 times less per sample when using the CP in 10*50
204 mm pieces). The CP still performed satisfactory, so therefore we consider it a possible option in
205 circumstances where budget is an issue.

206 The adjusted cut-off differed between the two countries: in Tanzania (NFS) it was adjusted to
207 84.6 % and in Pakistan to 69.0 %. This is due to the higher positive proportion in Pakistan, 80.0 %
208 versus 10.7 % in Tanzania. Previous studies in Tanzania have found a similar or slightly higher
209 prevalence, however, performed in animals of all ages (Kgotlele et al., 2016, Torsson et al., 2017, Muse
210 et al., 2012). In Pakistan, previous studies have found a similar prevalence or slightly lower (Zahur et
211 al., 2008, Zahur et al., 2011, Abubakar et al., 2017). In a high prevalent area, the sensitivity of a test
212 needs to be higher, to avoid a large number of false negatives. To increase the sensitivity in the used
213 ELISA the cut-off was lower in the high prevalent area (Pakistan) compared to the low prevalent area
214 (Tanzania). The final decision on the adjusted cut-off must depend on the aim of the test and where the
215 test is going to be used. When used for a screening purpose a higher cut-off is suggested to detect
216 potentially infected animals as early as possible, which is desirable during the eradication program. As
217 the prevalence in the population decreases so will also the positive predictive value of the test. To
218 increase the positive predictive value one could target the sampling to risk groups, use a test with a
219 higher specificity, or use an additional test and interpret the results in series. The optimum cut-off also
220 depends on the frequency distribution of the test variable in the healthy and diseased populations
221 (Thrusfield 2007). Moreover, even for a certain diagnostic test, the cut-off value is not universal and
222 should be determined for each region and for each disease condition (Pfeiffer, 2010, Habibzadeh et al.,
223 2016).

224 These results demonstrate that filter papers are an acceptable and cost-effective transport
225 method of whole blood samples for later use in serological analysis. Here we dipped the filter paper in
226 blood collected in a serum tube. In a field setting we would recommend to take the blood samples using
227 only an injection needle, letting the blood drip down on the filter paper (excluding the need of a serum
228 tube), which is then left to dry away from direct sunlight. Filter papers could be used, and perform well,
229 in low-income and large countries, where access to laboratory facilities is limited or available only at a
230 great distance and where the cold chain is difficult to maintain. It is however important to adjust the cut-
231 off value for a positive result when using filter papers, and as we have shown here by performing the
232 study in two different countries, in a low prevalent setting the cut-off may need to be adjusted to a higher
233 degree than in a high prevalent setting.

234

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