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COMPARATIVE EFFICACY OF SINGLE RADIAL HAEMOLYSIS TEST AND COUNTERCURRENT IMMUNOELECTROOSMOPHORESIS WITH MONOCLONAL ANTIBODIES-BASED COMPETITIVE ELISA FOR THE SEROLOGY OF PESTE DES PETITS RUMINANTS IN SHEEP AND GOATS

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Summary

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This project was conducted to investigate the comparative efficiency of competitive ELISA (cELISA), single radial haemolysis test (SRH) and countercurrent immunoelectroosmophoresis (CIEOP) for the diagnosis of *Peste des Petits Ruminants* (PPR) in sheep and goats. Serum samples from 198 sheep and 82 goats were collected from three different government livestock farms and all the samples were run simultaneously with three serological tests. The samples found positive for PPR antibodies through cELISA, SRH and CIEOP were 96 (34.2%), 55 (19.6%) and 67 (23.9%), respectively. Kappa statistics was used to evaluate the concordance between the laboratory-based test (cELISA) and field-based tests (SRH and CIEOP). Kappa statistics scores for cELISA vs SRH and CIEOP were 0.5851 (95% confidence interval 0.4848–0.6854) and 0.6668 (95% confidence interval 0.5733–0.7603), respectively, which indicate a "moderate" agreement between cELISA and SRH and "substantial" agreement between cELISA and CIEOP. SRH and CIEOP revealed a relative diagnostic sensitivity with cELISA of 54.1% and 64.5% and relative diagnostic specificity of 98.3% and 97.2%, respectively. The data suggested that for mass screening and control of PPR, these serological tests proved practical in the absence of cELISA since they have a high relative diagnostic specificity and a satisfactory relative diagnostic sensitivity.

Key words: cELISA, goats, peste des petits ruminants, serological tests, sheep

INTRODUCTION

The existence of peste des petits ruminants (PPR) has been recognized in Pakistan since 1991 (Amjad *et al.,* 1996) and is considered to be one of the main constraints in improving productivity of small ruminants in the regions where it is endemic. Pakistan is an agricultural country with a total population of sheep and goats, 25.5 and 61.9 million head, respectively (Anonymous, 2006). The disease causes heavy economic losses on the basis of mortality and morbidity losses through body wastage, poor feed efficiency, loss of meat, milk and milk products and off-

spring. Presence of PPR in the country can limit trade and export and there is loss of sheep/goat produced food for human consumption.

PPR is frequently confused with other diseases that present fever and grossly similar clinical signs, which has delayed its recognition in some countries. Two major clinical signs are mouth lesions and difficult breathing. However, mouth lesions could be a symptom of rinderpest, foot-and-mouth disease, bluetongue or contagious ecthyma; and the difficult breathing could be a symptom of pneumonic pasteurellosis or contagious caprine pleuropneumonia.

Because of the necessity to detect PPR amid a number of other acute diseases with grossly similar presenting signs, and to control persistent infections, laboratory confirmation is indicated. Detection of virus antigens relies on mostly standardized reagent kits, commercially available for agar gel immuno-diffusion test (AGIDT) and immunocapture ELISA (ICE). Mostly, ICE is preferred as it is more specific and quick (Munir *et al.,* 2008). However, it needs lot of equipment and the cost of ICE kit is also very high. Seropositivity is a good indication because animals infected with the PPR virus carry antibodies for life, with the development of a sustained antibody response.

Single Radial Haemolysis (SRH) test is not commonly used for the diagnosis of PPR but it has been reported to be specific, simple and sensitive by Bansal *et at.* (1986). Countercurrent immuno-electro-osmo-phoresis (CIEOP) is currently in use in Pakistan for the serology of PPR. This test is relatively rapid, inexpensive, simple and sensitive to identify animals infected with the PPR virus, although it is interpreted subjectively by visual reading of precipitation line curvature (Duraojaiye & Taylor, 1984).

ELISA has been established by the National Veterinary Laboratories (NVL), Islamabad, Pakistan as test method for the diagnosis of PPR. However, ELISA kits may not be used as a mass-screening test due to its high cost. So, the following study was performed to estimate the performance characteristics (Kappa values, relative diagnostic sensitivity and specificity) of SRH test and CIEOP in relation to a commercially available cELISA kit (BDSL, UK). It was also investigated whether the adaptation of SRH and CIEOP as a diagnostic test method for the control and diagnosis of PPR is feasible in Pakistan.

MATERIALS AND METHODS

Source of samples

Two hundred and eighty serum samples from 198 sheep and 82 goats were collected aseptically from different farms situated in different parts of the country, including the Sheep and Goat Research Center at Khariwala, District Layyah; Livestock Production and Research Institute, Bahadurnagar, District Okara, and Livestock Experimental Station, Qadarabad, District Sahiwal, Punjab, Pakistan. The serological screening was carried out at the National Veterinary Laboratory, Islamabad, Pakistan and the Department of Veterinary Microbiology, Faculty of Veterinary Science, University of Agriculture, Faisalabad, Pakistan. There was no vaccination history against PPR. The origin of samples is shown in Table 1. The serum samples were transported to the laboratories in coolers with cold packs and were then stored at –20 °C until used.

Table 1. Description of samples collected at three district government livestock farms

Source of sampling	Species	Total number of animals at farms	Number of samples collected*
Livestock Experimental Station, Qadarabad, District Sahiwal	Sheep	241	23
	Sheep	176	21
Livestock Production and Research Institute, Bahadurnagar, District Okara	Sheep	483	48
	Sheep	379	38
Sheep and Goat Research Centre at Khariwala, District Layyah	Sheep	503	50
	Sheep	82	08
	Sheep	105	10
	Goat	118	11
	Goat	54	5
	Goat	119	11
	Goat	314	30
	Goat	158	25

*Samples are collected with the rate of about 10% of the herd at three farms from different sheep and goat breeds.

Source of PPR virus

PPR virus was procured from the Tissue Culture Section, Veterinary Research Institute (VRI), Lahore, Pakistan.

Hyper-immune sera

Hyper-immune antiserum to PPRV was produced following the method described by Elhag & Taylor (1984) with some modifications, at Laboratory Animal House in the Department of Veterinary Microbiology, University of Agriculture, Faisalabad, Pakistan. Goats were inoculated three times at weekly intervals. They were inoculated subcutaneously with 2 mL of the virus ($10^{5.4}$ $TCID_{50}$/mL) on the first (zero) day, 2 mL of the virus intravenously accompanied by 2 mL of an emulsion of equal parts of virus and Freund's incomplete adjuvant given intramuscularly on day 7 and day 14. Serum was examined for PPR antibodies by AGPT and cELISA and stored at –20 °C until used.

Negative control serum

Serum samples found negative by cELISA were taken as negative control sera.

Serological Analyses

Competitive ELISA

Competitive ELISA was performed as laid down by Anderson *et al.* (1991), by using Biological Diagnostic Supplies LTD (BDSL, UK) kit.

Results were interpreted by fitting a multichannel spectrophotometric ELISA plate reader (BDSL, Multiscan, Finland) with an interference filter of 492 nm used to read the test. The results were expressed in terms of percents of inhibition (*PI*) by converting the optical density to *PI* according to the following formula:

$$PI\% = 100 - \left(\frac{\text{Mean OD of test wells}}{\text{Mean OD of C mab wells}}\right) \times 100$$

where OD is the optical density and Cmab refers to the MAb control.

Samples with *PI* values >50% were regarded as positive.

Single Radial Haemolysis Test (SRH)

Preparation of sheep erythrocytes. The blood from jugular vein was obtained aseptically in a sterilized test tube using sodium citrate (3.8%) as anticoagulant. The blood was centrifuged at 1500 rpm for 10 minutes. Plasma and buffy coat were decanted and three washings were given to packed cell volume, first with physiological solution and twice with phosphate buffered saline solution (pH 7.2).

Coating and sensitization of sheep erythrocytes. Coating and sensitization were performed as narrated by Ahmed *et al.* (1985). Packed sheep erythrocytes (0.75 mL) were mixed with 29.25 mL of phosphate buffered saline to prepare 2.5% red blood cell (RBC) suspension. Tannic acid solution 0.005% was prepared by adding 2.5 mg tannic acid in 50 mL of physiological saline. An equal volume of 2.5% RBC was mixed with 0.005% tannic acid solution and incubated for 10 min at 37 °C in a water bath. The cells were centrifuged at 1500 rpm for 10 min and the supernatant was discarded. The tanned RBCs were washed with phosphate buffered saline and resuspended in the same dilution to give 2.5% cell concentration.

The tanned sheep RBC were then sensitized with PPR antigen by mixing one volume of 2.5% tanned erythrocytes with one mL of phosphate buffered saline (pH 6.4) at 37 °C for 15 min. The sensitized erythrocytes were washed twice in phosphate buffered saline (pH 7.2) and packed cell volume was used within three hours.

Complement. Fresh guinea pig serum was used as a source of complement. For this purpose, three adult animals maintained at Laboratory Animal House in the Department of Veterinary Microbiology, Faculty of Veterinary Science, University of Agriculture, Faisalabad, Pakistan, were bled and blood was collected in a clean and dry screw capped test tubes. The test tubes were immediately shifted to refrigerator (4 °C) to avoid any abatement in complement titre and allowed to coagulate. The coagulum was poked at various points and the tubes were then centrifuged at 3000 rpm for 15 min. The serum was collected and preserved in convenient aliquots of 1 mL at –20 °C until used.

Serum inactivation. All serum samples were placed in water bath at 56 °C for 30 min to eschew the chances of non-specific reactions prior to use in the SRH test.

SRH test was performed as laid down by Bansal *et al.* (1986), yet with some modification, based on pilot studies made. Erythrocytes sensitized with PPR antigen (packed cell volume) were mixed in 0.2 mL amount with 17.4 mL of 1.0% noble agar in veronal buffer (pH 7.2), maintained at 46 °C in water bath. The fresh complement (2.4 mL) was aptly mixed with the above suspension at about 44–45 °C and the material was layered (2–3 mm thickness) onto the surface of clean microscopic glass slides, about 2.5 mL each. The poured slides were shifted to the refrigerator for at least 15 min, to allow proper solidification of the agar gel thin layer on glass surface. Filter paper discs (paper wicks) of about 6 mm diameter were soaked in to the serum samples that absorbed nearly 0.035 mL amount each. Three discs were applied to a single slide with 2 cm apart. One of the slides bore two control discs, one soaked in known positive serum and other in the known negative serum. The slides were transferred to refrigerator (4 °C) overnight for the adequate diffusion of sera into the agar-gel and then shifted to 37 °C in the humid chamber and observed at 4, 6, 8, 10, and 12 h.

The results were recorded by initially measuring the average diameters (mm) of haemolytic zones developed around the serum-impregnated discs and later computing out the relevant areas in square millimeters by employing the formula πr^2.

Countercurrent immunoelectroosmophoresis (CIEOP)

CIEOP was performed as described by Durojaiye & Taylor (1984). Agarose (Difco, USA) 1 g was used in 100 mL of 1.0 M tris borate EDTA buffer (pH adjusted to 8.0) and autoclaved at 1.1 kg/cm^2 pressure and 121 °C for 20 min.

A layer of agarose was prepared on microscopic slide by adding 3 mL of agarose. After solidification, six wells were punctured in the agar on each slide. There was a distance of 6 mm between two wells of a pair and 8 mm between adjacent pair of wells. The diameter of each well was about 4 mm. In the first pair, the well of anode side was filled with 10 µL of positive control serum and in the last pair; the well on anode side was filled with 10 µL of negative control serum. In the remaining four pairs of wells, the anode side wells were filled with 10 µL of test serum and all cathode side wells were filled with 10 µL of antigen.

Agarose slide containing serum and antigen as reactants was paced on a bridge in the electrophoresis bath containing 1.0 M tris EDTA buffer. The current of 10 mA per slide at 25 °C was allowed to pass for 30–60 min. After the time elapsed, the agarose was examined for precipitin line. This procedure was repeated with each serum sample, dispensing serum samples and PPR antigen in anode and cathode sides respectively.

Statistical analysis

The relative diagnostic sensitivity and specificity were calculated for both SRH and CIEOP using the cELISA as the reference standard. The concordance between three different serological diagnostic tests was determined using kappa statistics (Landis & Koch, 1977).

RESULTS

In the competitive ELISA, 96 of the 280 (34.2%) samples turned out positive. The samples found positive in the CIEOP and SRH were 67 (23.9%) and 55 (19.6%), respectively.

In the absence of a gold standard, the relative diagnostic sensitivities and specificities of the three tests were estimated using cELISA as the reference standard. It was estimated that SRH has relative diagnostic sensitivity and specificity with cELISA of 54.1% and 98.3% while CIEOP has relative diagnostic sensitivity and specificity with cELISA of 64.5% and 97.2%, respectively. The cross comparisons from the three different tests for the samples (n=280), are shown in Tables 2 and 3.

Table 2. Detection of PPR antibodies in the field sheep and goat sera by cELISA and SRH[a] based on 280 serum samples

cELISA[b]	SRH		
	Positive	Negative	Total
Positive	52	44	96
Negative	3	181	184
Total	55	225	280

[a] Relative sensitivity of SRH vs cELISA = 52 of 96 or 54.1%; relative specificity of SRH vs cELISA = 181 of 184 or 98.3%; kappa value = 0.5851; [b] the cut-off value for cELISA was set at *PI* of 50.0%.

Kappa statistics were applied to ascertain the concordance between the laboratory-based test (cELISA) and field-based

Table 3. Detection of PPR antibodies in the field sheep and goat sera by cELISA and CIEOP[a] based on 280 serum samples

cELISA[b]	CIEOP		
	Positive	Negative	Total
Positive	62	34	96
Negative	5	179	184
Total	67	213	280

[a] Relative sensitivity of CIEOP vs cELISA= 62 of 96 or 64.5%; relative specificity of CIEOP vs cELISA = 179 of 184 or 97.2%; kappa value = 0.6668; [b] the cut-off value for cELISA was set at *PI* of 50.0%.

tests (SRH and CIEOP). Kappa scores for cELISA vs SRH and CIEOP were 0.5851 (95% CI 0.4848–0.6854) (Table 2) and 0.6668 (95% CI 0.5733–0.7603) (Table 3), respectively. According to the criteria by Landis & Koch (1977), scores were interpreted as moderate agreement between cELISA and SRH and substantial agreement between cELISA and CIEOP.

DISCUSSION

The cross comparisons from the three different tests indicated that SRH had a relative diagnostic sensitivity and specificity with cELISA of 54.1% and 98.3%, while CIEOP had a relative diagnostic sensitivity and specificity with cELISA of 64.5% and 97.2%, respectively. These results indicated that although the cELISA was a superior test, the other two methods were competent and able to provide a practical diagnostic capability in modestly equipped laboratories that cannot perform ELISA. However, ELISA kits could hardly be used as a mass-screening test due to high costs. SRH test and CIEOP can overcome this problem, since in these tests, local vaccine antigen is used. This makes both of these assays candidates for use in regional laboratories charged with performing mass screening for PPRV.

The kappa test scores for cELISA vs SRH and CIEOP indicated a “moderate” agreement between cELISA and SRH and “substantial” agreement between cELISA and CIEOP. A previous study conducted by Singh *et al.* (2006) has examined the new cELISA as an alternative for commercial cELISA in 2488 sheep and goats in India. We have also performed kappa test on their data; and on a test population of 835 sheep and 1653 goats, a kappa of 0.9473 was found, indicating an “almost perfect” agreement between tests. Recently, a similar study has been conducted by Nussieba *et al.* (2008), comparing the tests for PPR antigen detection. They calculated kappa statistics of 0.4366 between AGPT and HA. Munir *et al.* (2009) have also applied the same kappa statistics to ascertain the concordance between cELISA, AGID and PIT.

The seropositivity detected by CIEOP in this study (23.9%) is in accordance with a study conducted by Durojaiye & Taylor (1984), in which they used CIEOP to diagnose PPR in Northern Nigeria. The seroprevalence detected by CIEOP (23.9%) is higher than SRH (19.6%) but less than cELISA (34.2%). This outcome is in harmony with Durojaiye & Taylor (1984), who stated that CIEOP is suitable for screening sera in laboratories lacking facilities for the neutralization test. Pan *et al.* (1972) found CIEOP to be rapid and accurate in the detection of antibody in sera of pigs infected with African swine fever (ASF) virus. They also reckoned CIEOP to be superior to complement fixation test and the AGPT in the detection of ASF antibodies. One of the main advantages of the CIEOP is its rapidity in producing results, as precipitin lines were

often visible after the test was run for thirty minutes. The CIEOP is simple to perform, requiring very small quantities of reagents. Results obtained suggest that CIEOP can be used for seroepidemiological studies for PPR antibodies in modestly equipped laboratories.

Lowest seroprevalence detected by SRH, although SRH have been reported to be specific, simple and sensitive by Bansal *et al.* (1986) but fresh complement is required to perform this test. Callow & Bear (1980) reported that SRH is a successful method for mass survey of many haemagglutinating viruses like avian influenza virus.

Serological tests are preferred for mass screening of populations. Their main limitation is the failure to detect antibodies, i.e. sensitivity. Nucleic acids amplification methods, such as polymerase chain reaction in conventional and real-time formats offer a greater sensitivity, but they are usually too expensive for routine diagnosis in many laboratories, particularly in developing countries. Such is also the case with *in vitro* isolation in cell culture, because of high operating costs, quality assurance issues and a lack of trained staff and suitable facilities. Rapid assays such as immunochromatographic or magnetic bead format for the detection of antigen or antibodies, which are simple to perform and interpret and can be performed on-site or close to the farm, offer more practical solutions in the developing world. However, such rapid tests for the diagnosis of PPR are not currently available. The development of such technologies using the precipitation line on gel principles in a rapid, cheap and accurate format would be of great assistance to disease control authorities in many developing countries.

In conclusion, the results of the present study added valuable preliminary information on the comparative efficiency of SRH and CIEOP, and indicated that these may be suitable for mass screening in modestly equipped laboratories in the developing world as alternative to the available expensive tests.

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REFERENCES

Ahmed, J., M. Ashfaque & M. D. Ahmad, 1985. Isolation and confirmation of infectious bronchitis virus in poultry. *Pakistan Veterinary Journal,* **5,** 107–109.

Amjad, H., Q. U. Islam, M. Forsyth, T. Barret & P. B. Rossitter, 1996. Peste des petits ruminants in goats in Pakistan. *The Veterinary Record,* **139,** 118–119.

Anderson, J., J. A. McKay & R. N. Butcher, 1991. The use of monoclonal antibodies in competitive ELISA for the detection of antibodies against rinderpest and peste des petits ruminants virus. In: Seromonitoring of Rinderpest throughout Africa, Phase I, Proceedings of the Final Research Coordination Meeting of the FAO/IAEA/SIDA/OUA/IBAR/PARC, Vienna, Austria, pp. 43–53.

Anonymous, 2006. Government of Pakistan: Agricultural Statistics of Pakistan, Ministry of Food, Agriculture and Livestock (Economics wing), Islamabad, 2005-06; pp. 196–197.

Bansal, R. P., R. C. Joshi, B. Sharma & U. Chandra, 1986. Reverse phase passive haemagglutination test for the detection of rinderpest antigen. *Tropical Animal Health and Production,* **19,** 53–56.

Callow, K. A. & A. S. Bear, 1980. A re-examination of the single radial haemolysis technique for the assay of influenza anti-neuraminidase antibodies in human sera. *Archives of Virology,* **65**, 25–36.

Duraojaiye, O. A. & W. P. Taylor, 1984. Application of countercurrent immuno-electro-osmo-phoresis to the serology of peste des petits ruminants. *Révue d'Elevage et de Médecine Vétérinaire des Pays Tropicaux,* **37,** 272–276.

Elhag, A. B. & W. P. Taylor, 1984. Isolation of peste des petits ruminants virus from the Sudan. *Research in Veterinary Science*, **36,** 1–4.

Landis, J. R. & G. G. Koch, 1977. An application of hierarchical kappa-type statistics in the assessment of majority agreement among multiple observers. *Biometry,* **33**, 363–374.

Munir, M., M. Siddique, A. Shehzad, S. Zohari & K. Stahl, 2008. Seroprevalence of antibodies to Peste des Petits ruminants at various governmental livestock farms of Punjab, Pakistan. *Asian Journal of Epidemiology*, **1**, 82-90.

Munir, M., M. Siddique & Q. Ali, 2009. Comparative efficacy of standard AGID and Precipitinogen inhibition test with monoclonal antibodies based competitive ELISA for the serology of peste des petits ruminants in sheep and goats. *Tropical Animal Health and Production,* **41**, 413–420.

Nussieba, A. O., M. E. A. Rahman, A. S. Ali & M. A. Fadol, 2008. Rapid detection of peste des petits ruminants (PPR) virus antigen in Sudan by agar gel precipitation (AGPT) and haemagglutination (HA) tests. *Tropical Animal Health and Production,* **40,** 363–368.

Pan, I. C., C. J. D. Boer & W. R. Hess, 1972. African swine fever: Application of immuno-electrro-osmophoresis for the detection of antibodies. *Canadian Journal of Comparative Medicine,* **36,** 309–316.

Singh, R. P., P. Saravanan, B. P. Sreenivasa, L. C. Shah, R. K. Singh & S. K. Bandyopadhyay, 2006. Comparison of diagnostic efficacy of a monoclonal antibody-based competitive ELISA test with a similar commercial test for detection of antibodies for peste des petits ruminants (PPR) Virus. *Veterinary Research Communications,* **30,** 325–330.

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