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Clinical and laboratory diagnosis of contagious caprine pleuropneumonia in Qassim region, Saudi Arabia: a comparative study

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Abstract. Diagnosis of contagious caprine pleuropneumonia (CCPP) in Saudi Arabia mainly depends on clinical signs and post-mortem findings, in addition to limited usage of latex agglutination test (LAT). In this study, a PCR method specific for detection of *Mycoplasma capricolum* subspecies *capripneumoniae* (*Mccp*) was used as a direct confirmatory method and to compare it with clinical signs, necropsy lesions and LAT. During the 2016-2017 year, samples of serum, pleural fluid, lung tissue and nasal swab were collected from 40 goats with clinical signs of CCPP, which were selected from goats brought to the veterinary clinic of Qassim University from 18 goat herds and nine localities. Epidemiological data revealed 34.1%, 27.8% and 81.6% morbidity, mortality and case fatality rates, respectively. At necropsy, 31 of 40 goats (77.5%) were found with lesions matching those of CCPP. Molecular findings supported the suitability and applicability of PCR as a reliable method to diagnose and confirm CCPP directly from clinical samples. The disease was confirmed by PCR in 35 goats out of 40 (87.5%), 15 herds out of 18 (83.3%) and in all localities. Sera of 32 goats (80%) were found positive by LAT. Four of the five goats and two of the three herds negative by PCR were also negative by LAT and necropsy examination. Therefore, PCR sensitivity was considered 97.2% (35/36). Compared to the claimed high specificity and sensitivity of the used PCR method, diagnosis of CCPP based on clinical signs was found less specific and necropsy examination and LAT were less sensitive. It was concluded that molecular detection of *Mccp* directly in clinical samples should routinely be used to confirm diagnosis of CCPP in the region of study, prevent economic impact of wrong diagnosis and to hasten control process.

INTRODUCTION

Contagious caprine pleuropneumonia (CCPP), caused by *Mycoplasma capricolum* subspecies *capripneumoniae* (*Mccp*) is one of the most severe and devastating diseases of goats in many countries of Africa and Asia, including Saudi Arabia (Radwan *et al.*, 1985; Thiaucourt & Bolske, 1996; Çetinkaya *et al.*, 2009). The disease is characterized by fever, coughing and respiratory distress associated with fibrinous pleuropneumonia, unilateral lung hepatisation and accumulation of pleural fluid in the thoracic cavity (Thiaucourt & Bolske, 1996). *Mccp* belongs to the *Mycoplasma mycoides* cluster, a group of six closely related mycoplasmas which are pathogenic to ruminants and share some phenotypic and genomic properties (Thiaucourt *et al.*, 1996; Pettersson *et al.*, 1998), sometimes leading to the incorrect diagnosis of CCPP (Erno, 1987; Adehan *et al.*, 2006). The outbreak of the disease usually follows the introduction of an infected animal into a herd of susceptible goats and a short period of contact is enough for successful transmission through coughing (Thiaucourt & Bolske, 1996; OIE, 2008).

Laboratory diagnosis of the disease does not amount to its importance in some countries, including Saudi Arabia. This is mainly due to insufficiency of laboratory facilities, experience and difficulty to isolate the bacteria. Although the disease is known since 1873 (McMartin *et al.*, 1980), the causative bacteria was isolated for the first time in 1976 (MacOwan, 1976). However, using suitable media and growth conditions, the isolation was possible from around 23 countries in Africa and Asia (Samiullah, 2013). Most recently, the disease was confirmed in Saudi Arabia by isolation of *Mccp* and molecular identification of isolates (El-Deeb *et al.*, 2017). Isolation is necessary to confirm CCPP, but recently, some PCR and real-time PCR methods (Woubit *et al.*, 2004; Lorenzon *et al.*, 2008; Ying *et al.*, 2011) have been developed and found specific and efficient in detection of *Mccp* and recommended for confirmation of clinical signs (OIE, 2014). Serological tests such as indirect haemagglutination, complement fixation and latex agglutination are used to detect the antibody response of goats to *Mccp* (Samiullah, 2013). LAT is claimed more sensitive and can be performed in field conditions with a prompt result (Cho *et al.*, 1976). If LAT is combined with typical clinical signs and necropsy, it can be considered confirmatory (Rurangirwa *et al.*, 1987; OIE, 2014). A competitive ELISA for CCPP, based on a previous blocking ELISA is developed and found highly specific (Peyraud *et al.*, 2014). However, it is not available commercially.

This study was carried out to compare between clinical diagnosis of CCPP, detection of antibodies against *Mccp* by LAT and direct detection of *Mccp* in clinical samples by PCR in terms of specificity and sensitivity.

MATERIALS AND METHODS

Samples and clinical examination

Samples used in this study were collected from goats brought to the veterinary clinic of Qassim University, central Saudi Arabia during the 2016-2017 year. For this work, 40 goats belonging to 18 herds were selected for their clinical signs of CCPP. These animals were either died at the veterinary clinic or euthanized because of their condition. Animals treated or recovered were not included. Clinical and post-mortem examinations were performed and findings were recorded. Sera, nasal swabs in transport medium (taken after proper cleaning of external nares), pleural fluids collected by sterile syringes and lung tissues mainly from the interface between consolidated and unconsolidated areas were collected aseptically. Samples were put on ice box and sent to the veterinary microbiology laboratory at the university, where they were kept at -20°C until analyzed. Data about history, morbidity and mortality rates were gathered from owners of these animals.

Serological examination (LAT)

The collected serum samples (n=40) were tested for the presence of antibodies against *Mccp* using CapriLAT kit (Animal & Plant Health Agency, Surrey, UK) as per manufacturer's instructions. Briefly, a spot of 20 µl of serum was carefully dispensed onto one of 6 reaction cells of a black card using a micropipette. Latex reagent was well shaken before use and 20 µl volume was carefully put next to the serum spot and gently mixed together by a wooden stick and spread out inside the reaction cell. The mixture was rocked for three minutes and any degree of agglutination was recorded as follows: strong clumping considered as (+++), clear agglutination as (++), fine agglutination as (+) and no agglutination as (–). Positive and negative control sera were run with every 3-4 cards.

Molecular detection of *Mccp* in clinical samples

DNA extraction

Bacterial DNA was extracted from lung tissues (n=40), pleural fluids (n=40) and nasal swabs (n=20) using DNeasy® Blood & Tissue Kit (Qiagen). Nasal swabs were swirled in 2 ml phosphate buffered saline

(PBS) and ultracentrifuged (ALC refrigerated centrifuge, USA) at 13,500 rpm for 20 min at 4°C and sediment was resuspended in 200 µl PBS. Pleural fluids as 1 ml volumes in microtubes were first centrifuged at low rate (1500 rpm for 10 min) to pellet eukaryotic cell debris (Lorenzon *et al.*, 2008) and supernatant was ultracentrifuged at 13,500 rpm for 20 min at 4°C and sediment was resuspended in 200 µl PBS. Proteinase K (20 µl) was added to pellet suspensions of swabs and pleural fluids, which were then put into shaking water bath at 56°C for 45 min with vortexing every 15 min. Then they were treated as cell culture and mycoplasma DNA was extracted, along with lung tissues according to the kit manufacturer's protocol. Concentration of DNA in each extraction was determined by NanoDrop 1000 (Thermo Fisher Scientific Inc, USA) and accordingly diluted in elution buffer (provided in the DNA extraction kit) or distilled water to 10 ng/µl.

Detection of *Mccp* by PCR

All the DNA extracts were tested by PCR. The PCR was performed in Techne thermocycler, UK. A PCR master mix (2X) (Thermo Fisher Scientific Inc., California, USA) was used. The *Mccp* specific primers used by Woubit *et al.* (2004) were used, which were obtained from Thermo Fisher Scientific Inc. Amplification was carried out in a 50 µl final volume obtained by mixing 18 µl nuclease-free water, 1 µl of each primer (400 nM), 25 µl of PCR master mix (2X) and 5 µl of the template. Nuclease-free water was used as negative control. The PCR cycling conditions were performed according to Woubit *et al.* (2004). Twelve microlitres of each PCR product were mixed with 3 µl of 5X DNA loading dye and electrophoresed in 1.5% agarose gel in TBE stained with ethidium bromide (0.5 µg/ml) at 100 volts for 80 min. A 50 bp DNA ladder (Cleaver Scientific, UK) was used as size marker. The gels were analyzed in a UV transilluminator (Dolphin Doc^{Plus} Gel Image System, Wealtec Bioscience Co., Ltd., Taiwan). A sample is considered positive when a band of the expected size (316 bp) is displayed and the negative control do not show any band.

RESULTS

Clinical diagnosis findings

According to the data, which routinely collected from animal owners visiting the veterinary clinic, morbidity, mortality and case fatality rates varied between localities and goat herds (Table 1). Out of 1021 goats from 18 herds, 348 (34.08%) showed clinical signs of CCPP and 284 (27.8%) were died, representing 81.6% case fatality rate.

The clinical signs were almost similar in most cases with slight variations. The encountered clinical signs were fever (40.5–41.5°C), anorexia, depression, severe respiratory distress in forms of dyspnoea, painful breathing, mouth breathing, frequent painful coughing and grunting. Death of many cases was observed within five days and few cases recovered.

Table 1. Morbidity, mortality and case fatality rates in different herds

Herd Sr.	Locality	No. of animals	Morbidity rate	Mortality rate	Case fatality rate
1-3	Aldawadmy	30, 200, 180	66.7, 30, 33.3	50, 25, 27.8	75, 83.3, 83.3
4-7	Melida	100, 10, 15, 12	30, 20, 66.7, 66.7	25, 20, 60, 41,7	83.3, 100, 90, 62.5
8-9	Oyon Algwah	5, 17	100, 70.6	80, 52.9	80, 75
10-11	Alfyelik	55, 37	21.8, 67.6	16.4, 48.6	75, 72
12	Ryad Elkhabra	6	100	66.7	66.7
13	Almuthneb	200	14.5	14.5	100
14-15	Alrabeaya	70, 25	11.4, 72	11.4, 62	100, 83.3
16-17	Alshyhia	20, 9	75, 88.9	50, 77.8	66.7, 87.5
18	Alras	30	66.7	50	75
Total		1021	34.08	27.81	81.60

In most cases (n=31, 77.5%), the post-mortem lesions were observed confined to the thoracic cavity, which were considered indicative of CCPP. The majority of them were with one lung affected, which is covered by thick layer of fibrin. The prevailing lesions encountered were: fibrinous pleuropneumonia, pleurisy, consolidation and hepatization associated with pleural adhesions and effusions in some cases, and hydrothorax and straw colored pleural exudates (Fig. 1).

Serological findings

Thirty-two out of 40 tested sera (80%) were found positive by LAT. Out of the reactive sera, 13 were strong positive (+++), 14 were of moderate agglutination (++) and five showed weak reaction (+). One of the animals positive by LAT had post-mortem lesions consistent with CCPP but negative by PCR. While, there at least four animals negative by LAT, but positive by PCR and three of them also positive at necropsy.

Molecular findings

Presence of *Mccp* in clinical samples was detected in 33 (82.5%) lung tissues, 26 (65%) pleural fluids and 4 (20%) nasal swabs (Fig. 2). Animals of 24 positive pleural fluids were also positive in lung tissues. The four animals positive in nasal swabs were also positive in lung tissues and pleural fluids. This means that the total positive cases by PCR were 35 (87.5%). At herd and locality levels, CCPP was diagnosed in 15 out of 18 herds (83.3%) and in all localities. One of the animals positive by LAT and its post-mortem lesions were consistent with CCPP was negative by PCR. The comparison of overall results of the three diagnostic tools used is shown in Fig. 3.

DISCUSSION

Definite diagnosis of contagious caprine pleuropneumonia is made either by isolation of the causative agent (*Mycoplasma capricolum* subspecies *capripneumoniae*) from clinical samples such as lung tissue and pleural fluid taken at necropsy or by specific molecular identification of *Mccp* directly in clinical samples (OIE, 2014). The fastidiousness and slow growth of the organism, inadequacy of laboratory facilities and lack of experienced personnel make isolation a difficult task (Thiaucourt *et al.*, 1996; Çetinkaya *et al.*, 2009). Recently, some PCR-based methods have been developed specifically to identify the DNA of *Mccp* in culture or directly in clinical samples and found highly specific and sensitive (Woubit *et al.*, 2004; Lorenzon *et al.*, 2008; Ying *et al.*, 2011). This study was carried out to identify and confirm the presence of *Mccp* directly in clinical samples using PCR (Woubit *et al.*, 2004) for the first time in Qassim Region, Saudi Arabia, and to compare it with latex

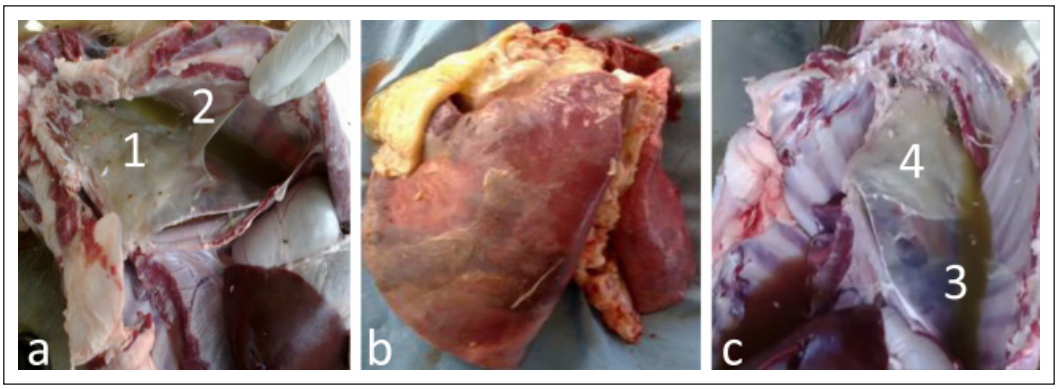

Figure 1. Postmortem findings of goats infected with contagious caprine pleuropneumonia: a) a lung covered with thick yellow coat of fibrin (1) and showing pleural adhesion (2); b) a lung showing congestion and red hepatisation and larger in size compared to the non-infected one; c) straw-colored pleural fluids (3). Note lung fibrin coat (4).

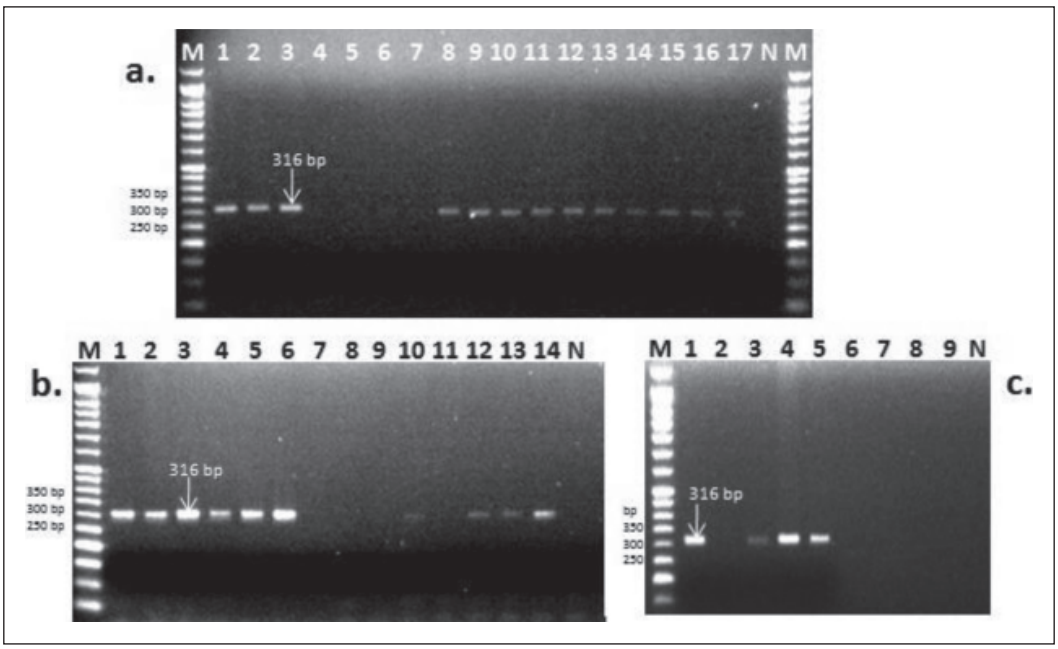

Figure 2. Gel electrophoresis of PCR products displaying a specific amplification of 316 bp of Mccp in, a) pleural; b) lung tissue and c) swab samples. Lane M, molecular weight marker (50 bp DNA ladder); numbered lanes are test samples; lane N, negative control.

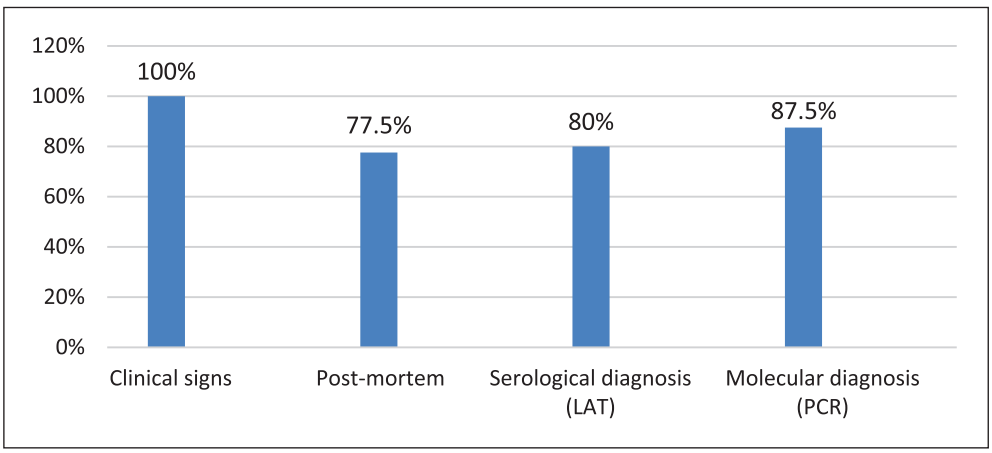

Figure 3. Comparison between clinical, serological and molecular positive results.

agglutination test and the mainly used method of diagnosis, clinical diagnosis (signs and post-mortem lesions). Very recently, El-Deeb *et al.* (2017) were able to isolate *Mccp* for the first time in Saudi Arabia and to molecularly identify it in cultures. The current study is the first one in this country to molecularly identify *Mccp* directly in clinical samples. Isolation takes several days, while direct detection would take only several hours (Hotzel *et al.*, 1996), which makes it preferable, especially in outbreaks and if isolation is already official in the country. In addition, direct detection is most likely more sensitive than isolation (Noah *et al.*, 2011; Ying *et al.*, 2011).

In the present work, CCPP was diagnosed by PCR in 87.5% (35/40) of the investigated goats, which were all selected because of their CCPP clinical signs; and in 15 of the 18 herds to which these goats belong. Four of the five negative animals, which belong to two of the three negative herds, were also negative by latex agglutination test and necropsy examination, and thus they were considered true-negatives. The fifth negative goat, which did not belong to any of the three negative herds, was positive by LAT and had post-mortem lesions consistent with CCPP, and thus it was considered a false-negative result by PCR. This PCR method is found slightly less sensitive when compared by a real-time PCR and another PCR methods (Lorenzon *et al.*, 2008; Ying *et al.*, 2011). Our finding indicated that PCR had at least 97% (35/36) sensitivity at the individual animal level, which is highly encouraging to use it as a routine diagnostic and confirmatory tool of CCPP; especially in outbreaks to hasten control process. The three negative herds were represented by one animal for each, which were erroneously diagnosed based on clinical signs only. Comparable sensitivity was reported before (Noah *et al.*, 2011; Ying *et al.*, 2011). The primers used for amplification of the target piece of *Mccp* DNA is found highly specific (Woubit *et al.*, 2004; Ying *et al.*, 2011), thus all cases found positive by PCR in the current study could be true positives. It is worth mentioning that measuring of DNA concentration in extracts of samples using NanoDrop 1000 spectrophotometer assisted us a lot to have this detection rate. DNA concentration of some samples was high and gave negative results, especially lung tissues, but they showed positive results after being diluted 1:3–1:50.

PCR identification of *Mccp* was highest in lung tissues (33/40), followed by pleural fluids (26/40) and lowest in nasal discharges (4/20). Similar finding was reported by Ying *et al.* (2011). However, some previous works found highest detection rate in pleural fluids (Noah *et al.*, 2011; Shah *et al.*, 2016). Large numbers of bacteria are expected in both pleural fluids and lung tissues from animals with acute cases of CCPP, unlike in nasal swabs (Bolske *et al.*, 1996). Two animals were positive in pleural fluids and not in lung tissues. This finding would necessitate testing of both sample types at least at the individual animal levels. High DNA concentration may give better results, but if very high, dilution is better (Ying *et al.*, 2011). The small volume of pleural fluid used for DNA extraction (1 ml) and may be large volume of elution buffer (200 µl) may contribute to lower detectability in pleural fluids.

The result of molecular detection of *Mccp* directly in the total number of clinical samples of all types (63%, 63/100) in the present study compared to isolation rate (7.8%, 55/700) reported before in this country (El-Deeb *et al.*, 2017), indicated that direct detection is much more sensitive than isolation.

Clinical signs and post-mortem lesions are the main methods routinely used for diagnosis of CCPP in the area of study, which are not sufficient to establish the diagnosis of CCPP (OIE, 2014). Diagnostic accuracy of clinical signs was found as maximum as 90% (36/40) according to the overall potential of other methods used in this work. Other diseases such as other pneumonic mycoplasmoses can display similar clinical picture and lead to erroneous diagnosis of CCPP (DaMassa *et al.*, 1992; Thiaucourt & Bolske, 1996). At necropsy examination, only 77.5% (31/40) of cases had lesions confined to the thoracic cavity and consistent with CCPP. One of them was found negative by PCR, but positive by LAT. As per overall PCR and LAT results (36/40), there are five cases could be considered false-negative by necropsy. These cases, which of atypical lesions may be mixed infection of *Mccp* and other organisms such as *Pasteurella* and other mycoplasmas (Radwan *et al.*, 1985; Noah *et al.*, 2011). Encountered lesions were in agreement with DaMassa *et al.* (1992), Wesonga *et al.* (1993) and Thiaucourt *et al.* (1996). Epidemiological data collected in this work revealed a high case fatality rate (81.6%) amongst affected goats. This high percentage was considered indicative of the disease and matching those reported for CCPP (Houshaymia *et al.*, 2002; OIE, 2009, Rurangirwa & McGuire, 2012).

Latex agglutination test demonstrated positive reactions of varying degrees (mainly strong to moderate agglutination) between antibodies raised against *Mccp* in sera of 32 goats (80%) against *Mccp* capsular polysaccharide (CPS) coating the latex beads. A part of its positive results, one animal was positive by PCR and negative at necropsy. This animal could be considered true-positive due to specificity of PCR and possibility of mixed infection that led to atypical post-mortem lesions. This means its results were highly specific if combined with specific necropsy lesions (Rurangirwa *et al.*, 1987). Specificity of *Mccp* LAT was found 100% in a study investigated mycoplasmoses in sera (n=260) of clinically affected goats (Shahzad *et al.*, 2012), when no single positive case was found. The antigen (CPS) of this test (CapriLAT) is a purified form of antigen, which might not cross-react even with closely related mycoplasmas (Rurangirwa *et al.*, 1987). Recently, Bertin *et al.* (2015) have reported the presence of the same polysaccharides at the surface (or secreted) of *M. capricolum* susbsp. *capricolum* and *M. leachii*. However, their presence in the area of study was not found documented (Radwan *et al.*, 1985) and, therefore it may not be important from a practical point of view. This test is claimed highly suitable for the rapid confirmation of outbreaks in the field, as agglutination is mostly triggered by IgMs, which are prevailing in early stages of the disease (Peyraud *et al.*, 2014). Compared with PCR and necropsy, at least four animals were negative by LAT. This may be attributed to the chronic stage of the disease in some animals (indicated by presence of adhesions). The test is considered useful early in the course of the disease (March *et al.*, 2000). Animals of this study were mainly from outbreaks in the area and most cases were in the acute stage. On the contrary, a recent study has found a correlation between the disease chronicity and LAT positivity (El-Manakhly & Tharwat, 2016). The test was found highly sensitive for *Mccp* strains (March *et al.*, 2000) and variable results were found for clinical investigations (Shahzad *et al.*, 2012; El-Manakhly & Tharwat, 2016).

It was concluded that molecular detection of *Mccp* directly in clinical samples was found highly sensitive and specific and should routinely be used for diagnosis of CCPP, especially in outbreaks to confirm the disease and to help control it rapidly. This study displayed a good agreement between necropsy, latex agglutination and PCR results, especially in specificity.

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