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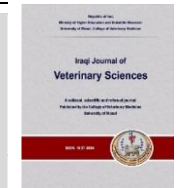

Assessment of multiplex PCR for detection of FMDV, BVDV, BTV, and possible coinfection with *Pasteurella multocida* in cattle

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Abstract

Multiplex polymerase chain reaction (mPCR) assay is a nucleic acid amplification method that is considered reliable and practical means for several pathogen detections in a single reaction, especially when multiple pathogens are suspected. In this study, a novel mPCR assay was validated for the detection of four notifiable diseases in cattle, including foot and mouth disease (FMD), Bovine viral diarrhea (BVD), Bluetongue (BT), and Hemorrhagic Septicemia (HS). The assay was operated in a two-step procedure. The first one was a reverse transcription of viral RNA, then mPCR of viral cDNA and bacterial DNA. The optimized mPCR was applied on blood (26) and vesicular epithelium (10) samples collected from 26 clinically infected animals from three governorates (Qalubia, Sharkia, and Gharbia). mPCR detected at least 10 pg of microbial nucleic acid extracted from the local isolates. The mPCR results showed that 22/26 (84.6%) of clinically infected animals were positively infected by single or dual infection. Mixed infection of FMDV and Pasteurella multocida was recorded in 11 animals (42.3%), while single FMDV infection was recorded in 5 animals (19.2 %). Single BVDV infection was detected in 5 animals (19. 2 %) and dual infection with FMDV in 1 animal (3.8%). Notably, BTV was not detected in any of the clinical samples. The assessed mPCR was a rapid, accurate, and sensitive test for diagnosing single and mixed infections in cattle and could be used to screen the notifiable diseases affecting cattle.

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Introduction

Animal notifiable diseases such as foot and mouth disease (FMD), Bovine viral diarrhea (BVD), Bluetongue (BT), and Hemorrhagic Septicemia (HS) are transmissible diseases that are required by law to be reported to government authorities and are considered the most important due to its ability to expand worldwide, and even its implications for the health of animal populations, wildlife and public health (1,2). FMD is a highly infectious transboundary disease that affects all cloven-hoofed animals and causes significant economic losses worldwide (3-5) due to decreased milk yield and meat production, the high mortalities in young animals, medication costs, and limitations on commercial animal traffic from endemic areas (6). Foot-and-mouth disease virus (FMDV) is an *Aphthovirus* belonging to the family *Picornaviridae* with genomic RNA of approximately 8.5 kb, which encodes 12 protein genes and a viral genome-related protein, including seven serotypes (O, A, C, South African Territories (SAT) 1, SAT2, SAT3, and Asia1) (7). As generally, FMDV does not cause mortalities in adult ages, it causes the animal to be

acutely immunosuppressed, leading to secondary bacterial complications by *Pasteurella multocida (P. multocida)*, and most of the mortalities were attributed to be due to Hemorrhagic septicemia (HS) infection (8). HS is an acute and fatal disease of cattle caused by the gram-negative bacterium *P. multocida.* The disease significantly impacts the livestock trade due to severe economic losses and is ranked as the most acute contagious disease in cattle (9). To restrict such bacterial infection, stress factors must initially be avoided, followed by rapid detection by PCR using universal genes and treatment of infected calves with appropriate antibacterial drugs after the sensitivity test (10). Another widespread notifiable viral disease of cattle is Bovine viral diarrhea (BVD) caused by the bovine viral diarrhea virus (BVDV). It is a positive sense that single-strand RNA belongs to the family *Flaviviridae* genus *Pestivirus*. BVDV is endemic in cattle populations worldwide and causes massive economic losses. There are 2 species of BVDV (BVDV1 and BVDV2) (11). BVD viruses are classified according to their ability to produce a cytopathogenic effect on cell culture into two distinct biotypes cytopathic (cp) and non-cytopathic (NCP) (12). The clinical affections of BVDV range from nonspecific signs such as fever, depression, inappetence, pneumonia, diarrhea, and Sores or ulceration in the mouth and gums may be present to highly fatal mucosal disease that occur in persistently infected calves with NCP BVDV and superinfected with cp (12). Rapid recognition and elimination of persistently infected animals are critical for effective control of the disease, and RT-PCR considers the most sensitive detection method (13). The same is true for BT, another notifiable disease of cattle, and RT-PCR is considered a very effective diagnostic method (14). BT is a disease resulting from infection with the Bluetongue virus (BTV), is economically significant, and can affect international trade and animal welfare (15). BTV is an arthropod-borne *orbivirus* (family *Reoviridea*), including 26 serotypes and responsible for mortality and trade limitation (16-18). BTV infections are inapparent or subclinical, especially in cattle in endemic areas. The clinical signs include high fever, oral lesions or ulcers, coronets, lameness, depression, weakness, and facial edema, as the clinical signs are more prominent in susceptible breeds of sheep, either in cattle or wild African ungulates (16). BTV genomic RNA is detectable in blood samples collected from infected animals for several weeks to months (14). Molecular techniques provide the potential for more efficient, rapid, and reliable ways to diagnose viral diseases directly from the source (19,20). PCR and reverse transcription-PCR are extensively practical techniques for DNA and RNA virus detection, respectively (21-26) and consider a rapid method that aids to give the suitable drugs in time to treat the diseased animals (27). The use of PCR in diagnostic laboratories is frequently restricted by its cost and, in some cases, the availability of sufficient sample volume. Multiplex PCR was developed to tackle these problems while also increasing the diagnostic potency of PCR (mPCR). The mPCR relates to using different pairs of primer sets to concurrently amplify different regions of the nucleic acid of the specimen with visualization of the amplicons by gel electrophoresis. The main advantage of this technology is that it reduces the number of separate reactions required as it detects multiple pathogens in a single specimen (28).

Egypt is endemic with several viral and bacterial pathogens that affect cattle and might be involved in similar symptoms that give rise to more complexity and difficulty for their diagnosis, especially in mixed infection. The conventional methods for diagnosing viral and bacterial diseases are inaccurate and time-consuming, causing delays in treatment to commence. The present study aimed to assess the mPCR technique to detect single and mixed infections of four notifiable diseases affecting cattle.

Materials and methods

Viruses, bacteria, and clinical suspected specimens

FMDV, BTV and BVDV, and *P. multocida* isolates were supplied by Animal Health Research Institute (AHRI), Dokki, Egypt. They were used in the standardization and validation of mRT-PCR. In addition, thirty-six blood and vesicular epithelium samples were collected from 26 clinically infected animals aged from 6 months to 4 years from different localities of three governorates (Qalubia, Sharkia, and Gharbia) between 2019 and 2021. Samples were collected from animals who suffered from fever over 40ºC, salivation, oral lesion, nasal discharge, cough (in some animals persist for 21 days), accelerated respiratory rate, diarrhea, and locomotors disturbance. These samples were preserved in a transport medium and were stored at -80°C until used according to OIE recommended protocols (29).

Oligonucleotide primers

Four pairs of primer sets were used to detect FMD, BVD, BT, and *P. multocida* in the mRT-PCR were adopted from previous studies (30-33), respectively**.** Primers were specifically amplified, targeting 5'UTR, UTR, NS3, and Kmt1 genes of FMD, BVD, BT, and *P. multocida*. All primers used in mPCR were needed to have the same annealing temperatures and lack dimmers or hairpin structures for mismatch avoidance (Table 1).

Viral and bacterial nucleic acid extraction and reverse transcription

The RNA of positive controls (FMDV, BVDV, BTV) and the clinically suspected specimen was extracted using QIA amp Viral RNA Mini Kit (Qiagen) cat. No. 52904 according to the manufacturer's instructions. While Bacterial DNA (*P. multocida*) was extracted using QIAamp DNA mini

kit (Qiagen) cat. No. 51304 according to the manufacturer's instructions from reference one and clinically suspected samples. ACCORDING TO THE MANUFACTURER'S INSTRUCTIONS, the RNA was reverse transcribed using Quanti Nova reverse transcription kit. Each reaction was performed in a 20 µl volume, which contains15 µl of the viral nucleic acid samples (5 µl from each virus), 1 µl reverse transcription enzyme, and 4 µl reverse transcription mix**.** The reactions were performed under the following conditions 25˚C for 3 min, followed by 45˚C for 10 min., then 85˚C for 5 min.

Establishment of multiplex PCR and its optimization

To optimize mPCR, different annealing temperatures (Ta), primer concentrations, extension times, and cycle numbers were tested. The mPCR products were analyzed by 1.5% agarose gel electrophoresis (34).

Table 1: Oligonucleotide primers used in multiplex PCR for detection of the target pathogens

Target	Gene	Sequence (5'-3')	Amplified fragment (bp)	Reference
FMDV	5'UTR	GCCTGGTCTTTCCAG GTCT CCAGTCCCCTTCTCAGATC	326	(30)
BVDV	UTR	GGGNAGTCGTCARTGGTTCG GTGCCATGTACAGCAGAGWTTTT	194	(31)
BTV	NS3	TCGCTGCCATGCTATCCG CGTACGATGCGAATGCAG	251	(32)
P. multocida	Kmt1	ATCCGCTATTTACCCAGTGG GCTGTAAACGAACTCGCCAC	460	(33)

Codes for mixed bases positions N: A/C/G/T; R: A/G; W: A/T.

Specificity and sensitivity of mPCR

The specificity of the mPCR was performed on FMDV, BVDV, BTV, and *p. multocida* with specific primers. Similar trials were used to distinguish possible cross-reaction of FMDV, BVDV, BTV, and *P. multocida* primers with RNA/DNA extracted from positive controls. The assay's sensitivity was assessed by making serial tenfold dilution of viral cDNA and bacterial DNA of control positives 1000, 100, 10, 1, 0.1 and 0.01 ng/µl. Later, the dilutions were used to determine the minimum detection limits of the mPCR methods.

Reproducibility of mPCR assay

The existing mPCR methodologies were conducted out as three separate mPCR assays at different points in time which used three different concentrations of positive controls to evaluate the reproducibility of the mPCR assay. The Nucleic acid was used as templates after dilution from 1000 ng to 0.01ng per µl, and one µl of each concentration of each target pathogen nucleic acid was mixed and amplified in mPCR.

Performing mPCR on extracted nucleic acids

The cDNA and DNA of 36 blood and vesicular epithelium samples were submitted for the previously optimized mPCR to detect the accused pathogen.

Determination of odds ratio and relative risk

The odds ratio (OR) is used to assess the association between the FMD virus and the presence or absence of coinfection risk factors and the incidence rate results**.** Relative risk is the ratio of the probability of an event happening in the exposed population to the probability of the event happening in the non-exposed population. It does not provide details about the actual risk of an event occurring but rather the higher or lower probability of risk in the exposed versus non-exposed group (35).

Statistical analysis

The results were statistically analyzed for the determination of odds ratio and relative risk by using the Chi-square test (two-tailed).

Results

Optimization of the mPCR technique

mPCR optimization showed that the annealing temperature at 50ºC, 1 µl of 20 pmol forward and reverse primer concentrations, 10 minutes extension time, and 35 cycles gave the optimum results. No primer dimers or nonspecific amplicons for tested pathogens were detected. The specific bands for FMDV, BVDV, BTV, and *P. multocida* were recorded at sizes 326bp, 194bp, 251bp, and 460 bp, respectively, as shown in (Figure 1).

Specificity and sensitivity of the mPCR method

The specificity appeared as specific PCR products were produced for each primer with no cross-reaction of FMDV, BVDV, BTV, and *P. multocida* primers; moreover, there were no unique amplicons in the lanes indicating negative controls. While the sensitivity of the assay revealed that the lower limit for detection (LOD) corresponded to 10

picograms (pg) for the nucleic acid extracted from the local viral and bacterial isolates as in (Figure 1).

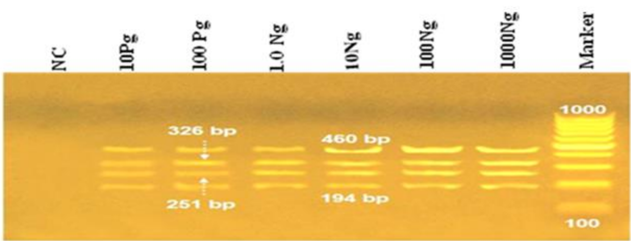

Figure 1: gel electrophoresis of multiplex PCR amplicons from 10-fold serially diluted cDNA/DNA extracted from local isolates of the four target pathogens. Bands at 460 bp for *P. multocida,* 326 bp for FMDV, 251 bp for BT and 194 for BVDV. Marker, DL 100 DNA Ladder molecular weight marker; Nc, negative control.

The reproducibility of mPCR assay

Testing the mPCR reproducibility proved that the technique, under various circumstances, could produce similar accuracy.

Evaluation of clinical samples

The optimized mPCR was applied to 36 samples collected from 26 clinically affected animals. The mPCR results showed that 22/26 (84.6%) of clinically affected animals were positively infected by single or dual infection. Mixed infection of FMDV and *P. multocida* was recorded in 11 animals (42.3%), while single FMDV infection was recorded in 5 animals (19.2%). Single BVDV infection was detected in 5 animals (19.2%) and dual infection with FMDV in 1 animal (3.8%). Notably, BTV was not detected in any of the clinical samples. (Table 2 and Figures 2-4).

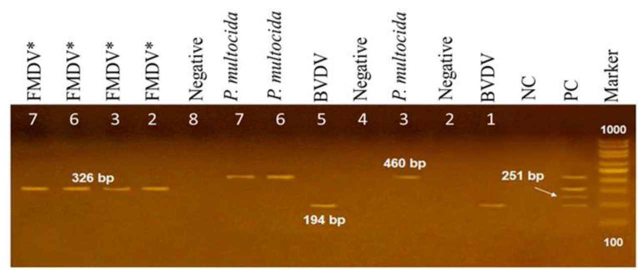

Figure 2: Agarose gel electrophoresis of 12 clinical samples (8 blood and 4 vesicular epithelium) collected from 8 animals identified by multiplex PCR. The number above each lane indicates the animal number. * Vesicular epithelium samples. Marker, DL 100 DNA Ladder molecular weight marker; PC, positive control; Nc, negative control. (460 bp for *P. multocida,* 326 bp for FMDV, 251 bp for BT and 194 for BVDV).

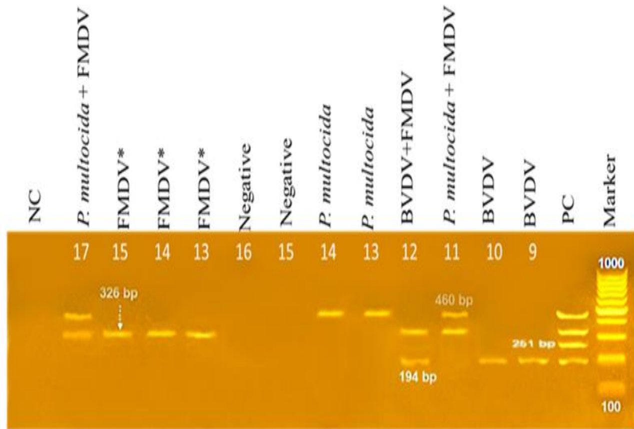

Figure 3: Agarose gel electrophoresis of 12 clinical samples (9 blood and 3 vesicular epithelium) collected from 9 animals identified by multiplex PCR. The number above each lane indicates the animal number. Vesicular epithelium samples. Marker, DL 100 DNA Ladder molecular weight marker; PC, positive control; Nc, negative control. (460 bp for *P. multocida*, 326 bp for FMDV, 251 bp for BT and 194 for BVDV).

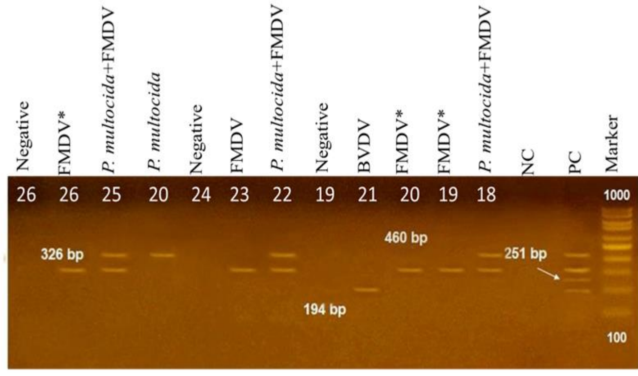

Figure 4: Agarose gel electrophoresis of 12 clinical samples (9 blood and 3 vesicular epithelium) collected from 9 animals identified by multiplex PCR. The number above each lane indicates the animal number. Vesicular epithelium samples. Marker, DL 100 DNA Ladder molecular weight marker; PC, positive control; Nc, negative control. (460 bp for *P. multocida*, 326 bp for FMDV, 251 bp for BT and 194 for BVDV).

Determination of odds ratio and relative risk

According to the OR, cases of FMDV coinfection with BVDV were 20 times less common than cases of FMDV infection alone. On the other hand, by Measuring the Relative Risk (RR), mixed infection of FMDV with *P. multocida* was 2.66 times more than FMDV infection alone (Tables 3 and 4).

Table 2: Incidence of FMDV, BVDV, BTV, and *P. multocida* in 26 clinically infected animals

Single infection				Mixed infection		+ve samples	Total
FMDV	BVDV	BTV	*P. multocida*	FMDV+ *P. multocida*	FMDV+BVDV		
5	5	0.0	0.0	11	1	4	26

Table 3: FMDV and BVDV coinfections

	FMDV Positive	FMDV Negative	Total	Relative Risk	Odds ratio	P-value
BVDV Positive	1	5	6	0.2	0.05	≤ 0.05
BVDV Negative	16	4	20			

OR = odds of BVDV infected animals / odds of non-BVDV infected animals. OR < 1.0, so BVDV infection act as a protective factor against FMDV infection. RR= Risk of FMDV in BVDV infected animals / Risk of FMDV in non-BVDV infected animals.

Table 4: FMDV and *P. multocida* coinfections

	FMDV Positive	FMDV Negative	Total	Relative Risk	Odds ratio	P-value
P.multocida Positive	11	0	11	2.5	Infinity	≤ 0.05
P.multocida negative	6	9	1٥			

OR = odds of *P. multocida* infected animals/odds of non- *P. multocida* infected animals. RR= Risk of FMDV in *P. multocida* infected animals / Risk of FMDV in non- *P. multocida* infected animals.

Discussion

The mPCR protocol was developed in this study for simultaneous detection of single and mixed infections in cattle. The developed assay permits early detection for proper reaction to the novel introduction of four notifiable diseases (FMDV, BVDV, BTV, and *P. multocida*) into a flock or country, limiting its spread and eventually achieving its eradication (1). Given its rapidity, specificity, and sensitivity, the mPCR is a valuable device for clinically diagnosing the mixed infections of animal DNA and RNA pathogens (36). Previous studies described mPCRs for detecting various DNA and RNA viruses in animals, proving that mPCR has high sensitivity and specificity (37-39).

The perfect eradication programs of FMDV are not dependent only on continuous screening for the disease, but additional screening could be applied for the presence of emerging viruses that may cause similar or unspecific clinical signs. Here, the FMDV assay was combined with BVDV and BTV as a specific detection system with *P. multocida* to detect a possible secondary bacterial infection, which was responsible for significant mortalities in FMDV outbreaks.

Several concerns attributed to mPCR include using several oligonucleotides in the same PCR-reaction as primer dimer, nonspecific reaction, amplification of specific target at the expense of others moreover; sensitivity reduction (40). The developed, validated multiplex PCR was characterized by reasonable sensitivity despite merging several oligonucleotides as 10 pg of nucleic acid could be detected in this assay as observed in local isolates and clinical specimens. Furthermore, the background screening system was not affected by the concurrent amplification of FMDV, BVDV, BTV, and or *P. multocida* genome; therefore, mixed infection was detected in 12/26 (46.1%) of affected animals.

The odds ratio (OR) revealed that cases of FMDV coinfection with BVDV were 20 times less common than cases of FMDV infection alone. It can be clarified as BVDV enters the oropharyngeal mucosa via inhalation or ingestion, and initial replication happens in epithelial cells that line the mouth or the airway (13,41) and results in epithelial necrosis and vacuolation of the basal stratum and spinosum stratum of the squamous epithelia of the tongue and nasopharynx (42), which is regarded as a common site of FMDV primary infection (43).

On the other hand, it was revealed that almost all *P. multocida* positive animals have mixed infection with FMDV, and the OR was infinite odds, indicating that cases of FMDV coinfection with *P. multocida* were more common than cases of FMDV infection alone. Also, the Relative Risk (RR) gave the same results as it was 2.66 times more likely to have *P. multocida* in FMDV infected animals than in FMDV negative animals. The FMDV caused immunosuppression, resulting in uncontrolled multiplication of *P. multocida*, resulting in HS outbreaks in buffalo and cattle with high mortality rates, particularly at the age of 12-15 months after FMDV infection, as occurred in Egypt in 2012 (44,45).

Conclusion

The newly validated multiplex PCR assay provides an efficient, sensitive, specific, and low-cost technique for relevant bovine pathogens and provides an early warning

system that rapidly detects FMDV, BVDV, BTV, and *P. multocida*.

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Conflicts of interest

The authors declare that there are no conflicts of interest regarding the publication of this manuscript.

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تقييم تفاعل البلمرة المتسلسل المتعدد للكشف عن فيروسات الحمى القلاعية والإسهال الفيروسي البقري واللسان الأزرق والعدوى المشتركة المحتملة بالباستوريلا مولتوسيدا في الماشية

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الخلاصة

يعد تفاعل البلمرة المتسلسل المتعدد إحدى طرائق تضخيم الحمض النووي التي تعد أكثر الوسائل موثوقية وعملية للكشف عن مسببات الأمراض في تفاعل واحد، لا سيما عند الاشتباه في وجود العديد من مسببات الأمراض. في هذه الدراسة تم التحقق من صحة اختبار تفاعل البلمرة المتسلسل المتعدد الجديد للكشف عن أربعة أمراض للماشية يجب الإبلاغ عنها وهي مرض الحمى القلاعية والإسهال الفيروسي البقري واللسان الأزرق وتسمم الدم النزفي. تم إجراء الاختبار في خطوتين، الأولى كانت النسخ العكسي للحمض النووي الريبي الفيروسي ثم تفاعل البلمرة المتسلسل المتعدد للحمض النووي الريبي الفيروسي والحمض النووي البكتيري. تم تطبيق التفاعل على (٢٦ عينة دم و ١٠ عينات نسيج حويصلي) جمعت من ٢٦ حيوانا مشتبه إصابته من محافظات (القليوبية والشرقية والغربية). أوضحت النتائج أن التفاعل كان قادرا على كشف ما لا يقل عن عشرة بيكوغرام من الحمض النووي الميكروبي المستخرج من العزلات المحلية .وكانت نتائج التفاعل أن ٢٢/٢٦ بنسبة ٨٤,٦% من الحيوانات المشتبه إصابتها مصابة بشكل إيجابي بالعدوى الفردية أو المزدوجة. تم تسجيل الإصابة المختلطة لمرض الحمى القلاعية مع باستوريلا مولتوسيدا في ١١ حيوان (٤٢,٣٪) بينما سجلت الإصابة الأحادية بمرض الحمى القلاعية في ٥ حيوانات (١٩,٢٪). تم الكشف عن إصابة أحادية لمرض الإسهال الفيروسي البقري في ٥ حيوانات (١٩,٢٪) وإصابة مزدوجة مع مرض الحمى القلاعية في حيوان واحد (٣,٨٪). والجدير بالذكر أن مرض اللسان الأزرق لم يتم اكتشافه في أي من العينات السريرية. كان تفاعل الذي تم تقييمه اختبارا سريعا ودقيقا وحساسا لتشخيص العدوى الفردية والمختلطة في الماشية ويمكن استخدامه لفحص الأمراض التي يجب الإبلاغ عنها والتي تؤثر على الماشية.