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Assessing the enhancing effect of some PCR additives in the diagnosis of Avian Influenza (H5) and Marek's disease viruses

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ABSTRACT

DNA amplification by polymerase chain reaction is usually challenged by PCR inhibitors. Those inhibitors affect PCR efficiency and generate PCR products of low yield or poor quality. The Egyptian poultry industry currently faces two destructive diseases [Highly pathogenic avian influenza (A/H5N1) and Marek's disease (MD) viruses]. There was necessity to improve the diagnosis of both viruses using real time PCR test. Six commercial PCR additives with different modes of actions (BSA, TMA-cl, 2-Pyrrolidinone, sulfolane, betaine and glycerol) were studied to evaluate their enhancing effect on PCR test over the routinely used PCR. The study results showed positive enhancement effect of the 10-fold amplification for BSA, 2-Pyrrolidinone, sulfolane, betaine and glycerol [(0.94, 0.76), (1.02, 0.82), (0.49, 0.51), (0.68, 0.59) and (0.39, 0.33)] in the detection of A/H5N1 and MDV treated samples, respectively. A clear inhibitor scavenging effect was accomplished in BSA treated samples. However, an obvious inhibitory effect was recorded for TMA-cl (-0.14, -0.07). Skillfully; 2-Pyrrolidinone showed the highest enhancement effect that could impact diagnosis of both viruses and improve PCR yield and detection level. The gained results recommend the routine use of different PCR additives either solely or in cocktails.

Keywords: A/H5N1; Marek's; rt-PCR; inhibitor; enhancer.

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INTRODUCTION

Although PCR has become one of the most leading techniques in molecular biology, PCR inhibitors usually possess negative influence on its power through affecting DNA amplification. The term PCR inhibitors is a general term that embraces all substances that can adversely affect PCR [24]. PCR inhibitors can undergo their effect through three potential Mechanisms: (i) direct binding with active sites in DNA polymerase to block enzyme activity [4]; (ii) interaction of the inhibitor with the DNA [29]; and (iii) competitive binding of some substances as calcium with DNA polymerase instead of magnesium or even depleting enzyme cofactors as performed by tannic acid [18]. Also, high GC content (more than 60%) in the target DNA sequence was reported to weaken the PCR amplification power [19].

On the other hand, the co-parallel amplification of nonspecific target can affect PCR efficiency. To overcome PCR inhibitors' adverse effects, the enhancing effect of several materials were studied. Their modes of action varied widely, where bovine serum albumin (BSA) was one of the most leading materials that could be added to the PCR mastermix in order to scavenge inhibitors [9]. On the other hand, raising PCR efficiency and specificity could be achieved by some materials as Tetramethylammonium chloride (TMA-Cl) [30], sulfolane and 2-Pyrrolidinone [5]. Moreover, betaine was proven to undergo isostabilizing effects through equalizing the contribution of GC- and AT-base pairing to DNA stability by stabilizing AT pairs [23]. Betaine also enhances the amplification of templates with varying GC content [12], expands the optimal range for MgCl2 concentrations, thus allowing the amplification of DNA samples of lower quality [28]. The osmoprotectant character of betaine adds another advantage as it enables *Taq* to resist denaturation [11]. Another group of PCR enhancers are polyhydroxyl alcohols, out of this group is glycerol which was reported to improve PCR specificity [20]. Also, ethylene glycol and 1,2-propanediol were more effective than betaine in the amplification of multible GC-rich human DNA sequences [32]. Another mode of action to enhance PCR could be carried out by nonionic detergents as Tween 20, NP-40 or Triton X-100, where they limit the adsorption of reagents to tube walls and overcome inhibitory effects of SDS [8]. The tissue lysing effect of Triton X-100 allowed the direct amplification of DNA from solid tissues without beforehand extraction [16].

In Egypt, the HPAIV H5N1 viruses are circulating since 2006 and caused great losses in all domestic poultry species [3], however endemic status was announced during 2008 [1].

Marek's disease is an infectious alphaherpes virus-induced lymphoproliferative disease that usually possess destructive pattern in commercial poultry. The disease caused severe economic losses in the Egyptian broiler, layer and breeder flocks. MDV not only affects the peripheral nervous system but also other tissues and visceral organs [10]. Both diseases showed progressive and destructive patterns among different poultry flocks. The current filed response and disease control are based mainly on rapid laboratory diagnosis using PCR test. However, the high variability of different samples types and quality that usually received from different cases could increase the possibility of harboring PCR inhibitors. This enforces the continuous demand for improving their diagnostic efficiency and sensitivity.

The aim of the current study was to evaluate the enhancing effect of six different PCR additives for the diagnosis of A/H5N1 and MD viruses in different poultry samples.

MATERIALS AND METHODS

Viruses

A representative classic HPAI A/H5N1 strain [(A/Chicken/Egypt/128s/2012), GenBank accession JQ858485] which represents clade 2.2.1 viruses in Egypt and a gallid herpesvirus 2 strain [(MDV/Egypt/F425/2016), GenBank accession KX272740] used in this study were obtained from the virus repository of the Reference Laboratory for Veterinary Quality Control on Poultry Production (RLQP), Giza, Egypt as representative for RNA and DNA viruses, respectively. Both viruses were titrated in spf embryonated chicken eggs (Nile SPF eggs, Koom Oshiem, Fayoum, Egypt) to be used as infection viruses. Both viruses were confirmed to be free of other biological contaminants, including avian influenza H9N2, avian leukosis subgroup J, infectious bursal disease virus, avian reo virus, infectious bronchitis virus, Newcastle disease virus, chicken infectious anemia, avian encephalomyelitis virus, adeno viruses, EDS76 virus, avian mycoplasma, and Salmonella spp.

Tenfold serial dilution of each challenge virus was done using 9-day-old (for A/H5N1) and 11-day-old (for MDV) specific pathogen-free (SPF) embryonated chicken eggs via allantoic sac (A/H5N1) and chorioallantoic sac (MDV). Five eggs were inoculated per dilution. The median egg infectious dose (EID50) was calculated based on the previously described formula [2].

Experimental Infection and sampling protocol

Two separate experimental infections were conducted in biosafety level III chicken isolators (animal facility) at RLQP, Egypt. The experiments were conducted according to Animal Health Research Institute guidelines concerning research ethics in animals. All experimental SPF chicks were reared under controlled hygienic environment, kept under daily monitoring and maintained with ad libitum feed. Before starting the experiments, tracheal swab samples were collected from A/H5N1 groups, and blood and spleen samples were collected from MDV groups. Samples of both groups were tested by their specific real time PCR to guarantee the freedom of the birds from any previous infection (before experimental viral inoculation).

Virological and molecular monitoring during the pre-challenge period confirmed that all the birds were free from A/H5N1 and MD viruses infection.

A/H5N1: At 4 weeks age, each bird out of 15 birds in each group was I/V inoculated with 100 µl of 10^5 EID50 pure H5N1 strain. At the 2^{nd} and 4^{th} days post inoculation (dpi), different tissue samples (spleen, pancreas, trachea and brain) in addition to tracheal and cloacal swabs and fecal samples were collected randomly from 5 birds. One g of each tissues and fecal samples were collected in 5 ml sterile PBS, minced probably, however the swab samples were collected in 1 ml sterile PBS. Samples were centrifuged at 3000 rpm for 20 min. at 4^{o}C to pellet the debris, then supernatants were collected for testing.

MDV: Each group harbored 15 chicks (1 day old) that were S/C inoculated with 100 µl of 10^5 EID50 of the pure infection strain. At the 14^{th} and 21^{st} days post infection (dpi), different samples (spleen, feather follicle, feces and buffy coat) were harvested randomly from 5 birds.

Real-time PCR assays

Samples recovered from A/H5N1 and MDV infected groups were subjected to viral RNA or DNA purification using QIAamp viral RNA Mini kit and QIAamp DNA Mini kit (Qiagen, GmbH, Hilden, Germany), respectively. Working concentrations of the different PCR additives were prepared (Table 1). Each sample was splitted into 7 identical parts and tested (Once without any treatment and once after treatment with each of the six PCR additives). Specific primers and taqman probes were used [H5LH1: ACATATGACTACCCACARTATTCAG, H5RH1: AGACCAGCTAYCATGATTGC and H5PRO: (FAM) TCWACAGTGGCGAGTTCCCTAGCA (TAMRA)] to amplify 151 bp of AI H5 haemagglutinin gene [17] and another panel [MDF: TGGGACGACGCAAATATGATG, MDR: AATGGTTCATTAGTAGAGCAGTTGGC and MD probe: (FAM) CATGGTTTGTCTTGGGC AGAGCATGTG (TAMRA)] to amplify 108 bp of the MDV glycoprotein (gD) gene [14] are listed in table 2. Quantitect probe rt-PCR kit (Qiagen, GmbH, Hilden, Germany) was used to detect and amplify specific RNA and DNA fragments. Reactions were performed and analyzed using an Agilent MX3005P rt-PCR machine (Santa Clara, CA, USA), where a thermal profile of 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C was adjusted for MDV assay. However, H5 one step rt-PCR assay started with RT step at 50°C for 30 min and then followed by 95°C for 15 min; and 40 amplification cycles (95°C for 10 sec, 54°C for 30 sec, and 72°C for 10 sec).

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Table 1: Different PCR additives used in the study

Related group	PCR additive	Proposed action	Final conc.	Source
Proteins	BSA	Scavenges inhibitors	6 µg/µl	Thermo scientific Cat. B14
Tetraalkylammonium salts	TMA-cl	Stabilizes A-T pairs	16 mM	Sigma Aldrich Cat. T3411
Amides	2-Pyrrolidinone	Promotes base pairing	120 mM	Sigma Aldrich Cat. 240338
Sulfones	Sulfolane	Optimization of high GC template amplification	0.5 M	Sigma Aldrich Cat. T22209
Compatible solutes	Betaine	Stabilizes A-T pairs, stabilizes DNA and lowers melting temperature	5 M	Sigma Aldrich Cat. B0300
Polyhydroxyl alcohols	Glycerol	Lower strand separation and primers annealing temperature	10%	Thermo scientific Cat. 17904

Table 2: Efficiency of the different PCR additives with A/H5N1 real time PCR.

Dpi	Type of Sample (n=5)	Untreated	BSA		TMA-cl		2-Pyrrolidinone		Sulfolane		Betaine		Glycerol	
		A	A	B(±SD)	A	B(±SD)	A	B(±SD)	A	B(±SD)	A	B(±SD)	A	B(±SD)
2nd	Kidney	2/5	2/5	0.9(±0.03)^{a}	2/5	0.1(±0.01)^{b}	3/5	1.05(±0.06)^{c}	2/5	0.7(±0.03)^{d}	2/5	0.6 (±0.08)^{d}	2/5	0.4(±0.03)^{e}
	Pancreas	2/5	2/5	1.04(±0.09)^{a}	2/5	0.17(±0.04)^{b}	3/5	1.6(±0.1)^{c}	2/5	0.8(±0.05)^{d}	2/5	1.1(±0.04)^{a}	2/5	0.6(±0.02)^{e}
	Trachea	3/5	3/5	0.9(±0.05)^{a}	3/5	-0.04(±0.03)^{b}	3/5	1.0(±0.04)^{c}	3/5	0.5(±0.03)^{d}	3/5	0.7(±0.05)^{e}	3/5	0.36(±0.04)^{f}
	Brain	1/5	1/5	0.97(±0.0)^{a}	1/5	-0.5(±0.0)^{b}	3/5	1.3(±0.0)^{c}	1/5	0.5(±0.0)^{d}	1/5	0.61(±0.0)^{d}	1/5	0.62(±0.0)^{d}
	TS	3/5	3/5	0.39(±0.03)^{a}	3/5	-0.2(±0.14)^{b}	3/5	1.1(±0.06)^{c}	3/5	0.5(±0.02)^{d}	3/5	0.9(±0.09)^{e}	3/5	0.43(±0.05)^{d}
	CS	1/5	2/5	1.4(±0.0)^{a}	1/5	-0.06(±0.0)^{b}	2/5	1.0(±0.0)^{c}	1/5	0.4(±0.0)^{d}	1/5	0.8(±0.0)^{e}	1/5	0.26(±0.0)^{f}
	Feces	1/5	2/5	1.3(±0.0)^{a}	0/5	-	1/5	0.5(±0.0)^{b}	1/5	0.24(±0.0)^{d}	1/5	0.67(±0.0)^{b}	1/5	0.39(±0.0)^{d}
4th	Kidney	4/5	4/5	0.9(±0.02)^{a}	4/5	0.06(±0.08)^{b}	5/5	1.3(±0.09)^{c}	4/5	0.4(±0.04)^{d}	4/5	0.5(±0.04)^{d}	4/5	0.3(±0.03)^{e}
	Pancreas	4/5	4/5	0.9(±0.09)^{a}	4/5	-0.28(±0.2)^{b}	5/5	1.3(±0.05)^{c}	4/5	0.7(±0.05)^{d}	4/5	0.8(±0.05)^{d}	4/5	0.5(±0.04)^{e}
	Trachea	4/5	4/5	1.1(±0.06)^{a}	4/5	0.2(±0.09)^{b}	5/5	1.47(±0.06)^{c}	5/5	0.5(±0.03)^{d}	5/5	0.7(±0.04)^{e}	5/5	0.35(±0.02)^{f}
	Brain	4/5	4/5	0.7 (±0.06)^{a}	4/5	-0.15(±0.1)^{b}	5/5	0.86(±0.05)^{c}	4/5	0.3(±0.04)^{d}	4/5	0.7(±0.03)^{d}	4/5	0.3(±0.05)^{d}
	TS	4/5	4/5	0.58(±0.04)^{a}	4/5	-0.15(±0.1)^{b}	5/5	0.4(±0.02)^{c}	5/5	0.3(±0.05)^{d}	5/5	0.6(±0.05)^{e}	5/5	0.1(±0.02)^{f}
	CS	3/5	4/5	0.88(±0.07)^{a}	2/5	-0.12(±0.03)^{b}	4/5	0.79(±0.04)^{c}	3/5	0.5(±0.03)^{d}	3/5	0.5(±0.03)^{d}	3/5	0.46(±0.05)^{d}
	Feces	3/5	4/5	1.2(±0.1)^{a}	2/5	-0.81(±0.15)^{b}	4/5	0.6(±0.06)^{c}	3/5	0.4(±0.06)^{d}	3/5	0.6(±0.05)^{c}	3/5	0.35(±0.04)^{d}
Average 10-fold amplification			0.94		-0.14		1.02		0.49		0.68		0.39	

A: No. of positive/tested samples.

B: Mean10-fold amplification over the untreated samples was calculated for positive samples only according to the theory that tells that a 10–fold amplification should take 3.32 cycles (Agilent, 2012) [2].

+ value; indicate positive enhancement over the untreated samples.

-Value; indicate inhibition effect under the untreated samples.

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Table 3: Efficiency of the different PCR additives with MD real time PCR.

Dpi	Type of Sample (n=5)	Untreated	BSA		TMA-cl		2-Pyrrolidinone		Sulfolane		Betaine		Glycerol	
		A	A	B(±SD)	A	B(±SD)	A	B(±SD)	A	B(±SD)	A	B(±SD)	A	B(±SD)
14th	Spleen	3/5	3/5	0.8(±0.05)^{a}	3/5	0.05(±0.02)^{b}	3/5	1.03(±0.07)^{c}	3/5	0.6(±0.06)^{d}	3/5	0.66(±0.04)^{d}	3/5	0.34(±0.04)^{e}
	Feather follicle	3/5	3/5	0.9(±0.05)^{a}	3/5	0.3(±0.06)^{b}	3/5	1.0(±0.02)^{c}	3/5	0.6(±0.04)^{d}	3/5	0.65(±0.05)^{d}	3/5	0.37(±0.04)^{b}
	Buffy coat	3/5	3/5	0.6(±0.02)^{a}	2/5	-0.3(±0.0)^{b}	3/5	0.6(±0.04)^{a}	3/5	0.3(±0.02)^{c}	3/5	0.4(±0.02)^{d}	3/5	0.14(±0.04)^{e}
	Feces	2/5	3/5	1.14(±0.06)^{a}	1/5	-0.37(±0.0)^{b}	2/5	0.97(±0.01)^{c}	2/5	0.7(±0.06)^{d}	2/5	0.83(±0.01)^{d}	2/5	0.56(±0.03)^{e}
21st	Spleen	4/5	4/5	0.58(±0.04)^{a}	4/5	0.09(±0.02)^{b}	4/5	0.7(±0.01)^{c}	4/5	0.55(±0.04)^{a}	4/5	0.63(±0.03)^{a}	4/5	0.34(±0.03)^{d}
	Feather follicle	4/5	5/5	0.4(±0.02)^{a}	4/5	0.06(±0.02)^{b}	5/5	0.9(±0.04)^{c}	5/5	0.6(±0.04)^{d}	5/5	0.64(±0.03)^{d}	5/5	0.33(±0.04)^{a}
	Buffy coat	4/5	4/5	0.73(±0.02)^{a}	4/5	0.12(±0.02)^{b}	4/5	0.6(±0.04)^{c}	4/5	0.36(±0.01)^{d}	4/5	0.4(±0.03)^{d}	4/5	0.3(±0.03)^{d}
	Feces	3/5	4/5	0.93(±0.03)^{a}	3/5	-0.5(±0.08)^{b}	4/5	0.7(±0.02)^{c}	3/5	0.34(±0.05)^{d}	3/5	0.46(±0.06)^{d}	3/5	0.24(±0.02)^{e}
Average 10-fold amplification			0.76		-0.07		0.82		0.51		0.59		0.33	

A: No. of positive/tested samples.

B: Mean10-fold amplification over the untreated samples was calculated for positive samples only according to the theory that tells that a 10–fold amplification should take 3.32 cycles (Agilent, 2012) [2].

+ value; indicate positive enhancement over the untreated samples.

-Value; indicate inhibition effect under the untreated samples.

Different lowercase letters (a, b, c, d, e, f) on the right-hand side in a row denote the presence of statistically significant differences ($P < 0.05$) between different PCR enhancer treatment in Mean 10-fold amplification. Different letters in a column did not reflect the presence of any statistical differences.

For both tables 2 and 3: Different lowercase letters (a, b, c, d, e, f) on the right-hand side in a row denote the presence of statistically significant differences ($P < 0.05$) between different PCR enhancer treatment in Mean 10-fold amplification. Different letters in a column did not reflect the presence of any statistical differences.

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Measured parameters and statistical analysis

The 10-fold amplification of viral RNA/ DNA loads in different treated samples were determined in comparison to untreated PCR results. In addition, the number of positive samples for each type of samples in accordance to the type of PCR enhancer treatment used.

Data from both experiments were collated, and statistical analysis was conducted using SPSS 21 (IBM Corp., Armonk, NY, USA). For each experiment, the variations within and between the 10 fold amplification results of the different PCR enhancer were compared using one way ANOVA and Tukey's multiple comparison test as appropriate, and $P < 0.05$ was considered statistically significant.

Mean10-fold amplification over the untreated samples was calculated for positive samples only according to the theory that tells that a 10–fold amplification should take 3.32 cycles [2].

RESULTS

Post-infection monitoring

Visible characteristic signs were observed at the 3rd dpi in the A/H5N1 groups. At the 4th dpi, 3/5 birds died with clear A/H5N1 PM lesions. On the contrary, no birds died in the MD infected groups till the end of monitoring period and collecting the samples. However, 4 birds showed depression, emaciation and swollen feather follicles starting from the 19th dpi. At the 21st dpi, all the remaining birds showed the same signs.

Efficiency of the different PCR additives

In the current study, variable results were encountered for the six additives tested (Tables 2 and 3), (Fig 1 and 2). Some of them enhanced the real time PCR efficiency while others not only didn't enhance efficiency but also had an inhibitory effect on PCR.

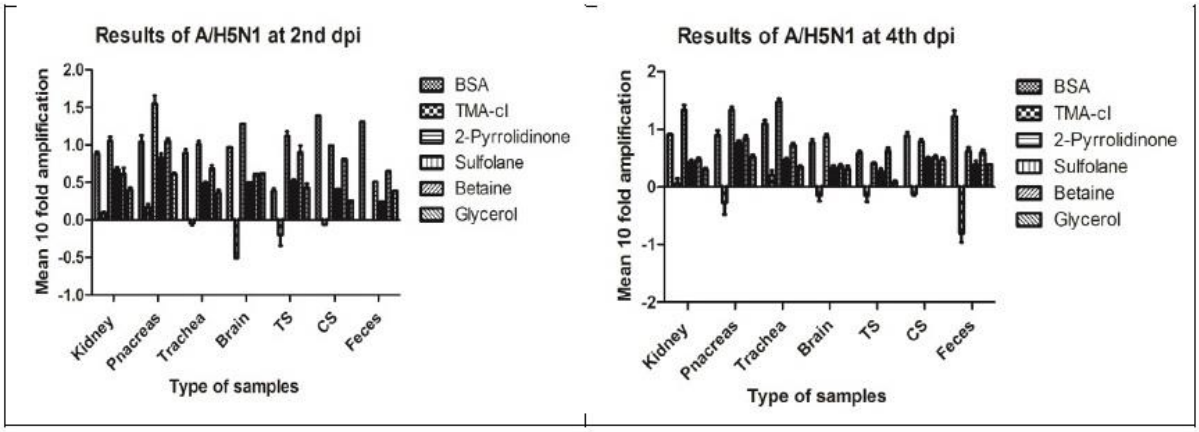

Fig.1 Mean 10-fold amplification (Mean ±SD) for different A/H5N1 treated samples in comparison to untreated samples

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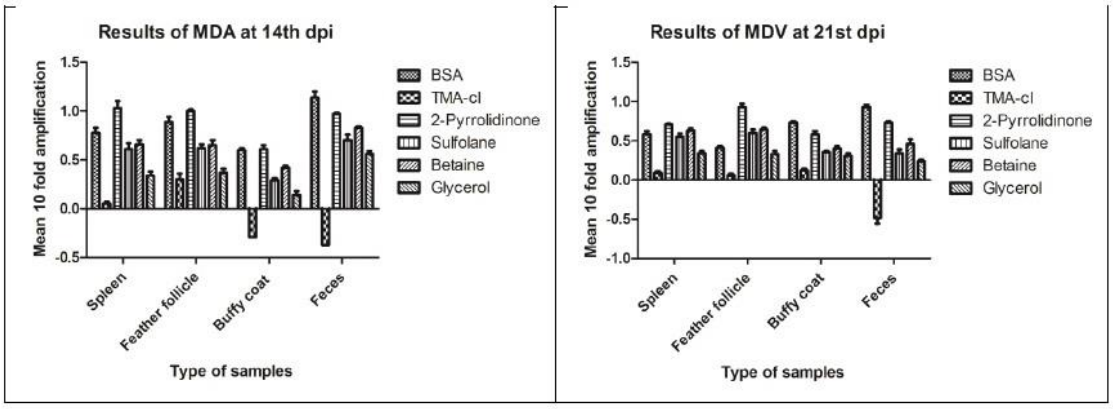

Fig.2 Mean 10-fold amplification (Mean ±SD) for different MDV treated samples in comparison to untreated samples

All A/H5N1 samples types showed positive results. The enhancing effects of 5 PCR additives (All additives except TMA-cl) were obvious, where 2-Pyrrolidinone and BSA markedly enhanced PCR sensitivity.

Collectively, thirteen negative samples results turned to positive after treatment with 2-Pyrrolidinone. A lower enhancing effect was recorded for BSA treated samples as 5 samples showed positive results after treatment.

On the other hand, TMA-cl showed clear inhibitory effect as it turned 2 positive samples to negative. The average 10-fold amplification over the untreated samples was -0.14 and -0.07 for the AI and MD samples, respectively.

DISCUSSION

PCR inhibitors represent continuous challenge that usually cripple any development in the PCR test. To overcome such PCR damage, six commercial PCR additives were evaluated for there PCR enhancing effect.

The enhancing effect of BSA was recorded mainly in cloacal swab and fecal samples. Such samples were usually reported to have high load of PCR inhibitors [7]. In their report, the inhibitors scavenging effect of BSA in feces was recorded [15], where two proteins (BSA and T4 Gene 32 Protein) had a relieving effect on the PCR inhibitors in extracts from feces, freshwater and marine water.

Although BSA performed the same inhibitor relief action in fecal samples during the detection of MD virus, this effect was somewhat lower in buffy coat samples in both 14^{th} and 21^{st} dpi samples as the 10-fold amplification was only 0.67 in comparison to 1.04 in fecal samples of the same birds. A possible cause of such decreased potency is the presence of high conc of leukocyte DNA, which was reported to be a very strong PCR inhibitor [31]. Buffy coat samples may also have traces of heme which is a potent PCR inhibitor. In his study, the inhibition from hemin could be relieved by BSA, however its degradation product "bilirubin" was not relieved by BSA. Hemin was over 1,000-fold more potent than bilirubin [15]. He suggested finally that BSA can overcome only one mechanism of hemin PCR inhibition, however a second mechanism, in common with bilirubin is not affected by BSA. Another explanation of decreased scavenging power of BSA in buffy coat samples is that these samples may contain some traces of EDTA anticoagulant which was also proved to decrease the anti-inhibitory power of BSA.

However, yet BSA still have the most potent effect in enhancing PCR efficiency in buffy coat samples as its 10-fold amplification (0.67) was still higher than those of all PCR additives evaluated in the study, which was somewhat encouraging because blood samples are extensively used for the molecular diagnosis of microbial infections [21].

On the other hand, the feather samples showed higher positive incidence in the 21st day post infection than that of buffy coat samples. This was declared when they studied MD disease in different tissues and reported that MDV DNA load in blood samples was lower than in solid tumors and feather pulp samples [6].

The best shift in PCR sensitivity was recorded for 2-Pyrrolidinone treated samples, where it showed 1.02 and 0.82 improvement of the average 10-fold amplification for the AI and MD samples, respectively. This may be related to the proposed A-T pairs stabilization action of 2-Pyrrolidinone. This rising in sensitivity may be reasonable as the AT% of AI and MD amplified fragments were 60% and 56%, respectively. Such DNA amplification improvement for 2-Pyrrolidinone was recorded by other authors who recommended it as the most effective low molecular weight amide in the enhancement of PCR amplification and specificity [5]. They also stated that the superior overall performance of 2-Pyrrolidinone compared to other acyclic amides may be due to its greater affinity for the grooves of the double-stranded template.

The same mode of action (Stabilization of A-T pairs) was recorded for betaine by some authors as they mentioned that betaine exerts its isostabilizing effect without changing the conformation of double-stranded DNA from the B form [23]. This may be the mode of action that enabled betaine to show a promising enhancing effect in the current study (0.59 and 0.68 enhancement of the average 10-fold amplification for the AI and MD samples, respectively), it also leaded to a positive result in two negative feather follicle samples. Controversially, the DNA-helix-destabilizing action of betaine cannot work alone for the PCR-enhancing effect [13], this may prompt other mechanisms as the thermostabilization effect of *Taq* polymerase [26], or expanding the optimal range for Mgcl2 concentrations [28] as supplementary factor conferring the enhancing feature.

Furthermore, a trustee enhancing effect was obtained for sulfolane. But this effect was not so high. This may be because sulfolane's enhancing effect was usually achieved with high GC templates [5].

Although a very low enhancing effect was obtained for some TMA-cl treated samples in the current study (which may be due to the A-T stabilizing effect), unexpected results were obtained for TMA-cl as it not only didn't alter the results of any of the negative samples, but it also turned 2 positive samples to negative. The average 10-fold amplification over the untreated samples was -0.14 and -0.07 for the AI and MD samples, respectively. An inhibitory effect of TMA-Cl and DMSO with several tested primers, including a primer for GAPDH and reverse primer for CD4 was previously reported [25]. They reported also that oligonucleotide primer-induced SGI fluorescence was completely inhibited when both TMA-Cl and DMSO were added together.

Glycerol showed a lower enhancing effect than other additives (0.39 and 0.33 for the AI and MD samples, respectively). A possible explanation was reported as that the high concentrations of glycerol may lead to inhibitory effects on *Taq* polymerase [27], such inhibitory effect motivated some authors to recommend using glycerol in a cocktail with BSA and DTT, the later substances might protect polymerase activity from the possible glycerol inhibitory effect [20].

Lastly, the study showed valuable progression of the PCR efficiency for almost all the additives except for TMA-cl. Greatest shifts of PCR sensitivity were encountered for BSA and 2-Pyrrolidinone. Some substances (betaine, sulfolane and glycerol) showed moderate enhancing effect suggesting using them in a cocktail that may have better enhancing effect through relieving their possible inhibitory effect.

In conclusion, the study set forth the enhancing effect encountered in the Egyptian endemic highly pathogenic AI (H5) and MDV real time PCR after treatment with different PCR additives that showed obvious shifts in PCR sensitivity and/ or inhibitors scavenging effect in different types of biological samples. The study strengthens the troubleshooting tools for week positive PCR results.

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