USING MITOCHONDRIAL CYTOCHROME β GENE FOR AUTHENTICATION OF MEATS FROM DIFFERENT ANIMAL SOURCES

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Quality of meat is of concern to researchers, consumers, retailers and governmental control authorities at all steps of the production process. Nevertheless, meat can be attractive target for adulteration in many ways and one very obvious kind is to illegally sell cheaper meat as meat from more profitable and desirable species.

In Egypt, where population raises the demand for meat products escalates, and, thus, they become the most highly-priced commodities. These encourage economic gain-oriented meat species adulteration especially in ground and comminuted products. One common type of adulteration in Egypt is to add inferior quality meat with superior one (e.g. mixing chevon for mutton, and buffalo and camel meats for cattle meats). Another type is to add deliberately meat from objectionable species with meat commodities commercially available in this country (e.g. mixing pork for mutton, and donkey meat for meats from cattle, buffalo and camel). Laws of Islam prohibit Muslims from eating pork and domesticated donkey meat (Mangar, 2015). The recent donkey meat scandal in Egypt (Beer, 2015) put consumers in red alert vis-à-vis both the presence of *'haram'* (not allowed) meat in their food and the indication that the meat they eat has been processed under non-sanitary conditions, thus representing potential risk to their health.

DNA-based techniques are regarded as the most pertinent methods for species identifications. DNA is existent in nearly all tissue types of an individual, stable under different circumstances (e.g. freezing, salting, drying, cooking and manufacturing) and permits for differentiating even very closely-related species due to the diversity afforded by the genetic code.

In spite of their well-known utility in permitting explicit species identification in some Egyptian research laboratories (Abd El-Razik *et al*., 2007; Abdel Rahman *et al*., 2009; Abd El-Nasser *et al*., 2010; Zahran and Hagag, 2015), DNA techniques have not yet been employed by public quality control laboratories and inspection services authorities to verify species composition of traditional meat articles available in this country.

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As the application of polymerase chain reaction (PCR) seems to give the most satisfactory results (Teletchea *et al*., 2005), the objective of this study was to use simplex and multiplex PCR assays with species-specific oligonucleotide primers targeting mitochondrial *cytochrome-b* (*mtCyt-b*) gene for the detection of two '*haram*' meat species, namely pig and donkey, and five *'halal*' (allowed) meat species, namely, goat, sheep, cattle, camel and buffalo.

MATERIAL AND METHODS

1. Raw meat sample collection and preparations

In total, 35 raw meat samples were sampled, representing seven species, *viz* goats, sheep, pigs, cattle, camels, buffaloes and donkeys (Table 1). All the collected samples were transferred, under ice chilled condition (4°C) and stored frozen (-20°C) without removing their containers or packages.

The samples used for qualitative detection of meat adulteration included two categories, the first was prepared in single-species and the second included samples made up with equally mixed meat of small species, large species and all small and large species.

2. Oligonucleotide primers designing

Mitochondrial *Cytochrome-b (mtCyt-b)* gene sequences for the seven-investigated species were retrieved from GenBank database (NCBI, 2016); the accessions are JX286582.1 for *Capra hircus* (*goats*), JX235881.1 for *Ovis aries* (sheep), NC_000845.1 for *Sus scrofa* (*pigs*), GU947009.1 for *Bos taurus* (*cattle*), JN632608.1 for *Camelus dromedaries* (*camels*), NC_006295.1 for *Bubalus bubalis* (*buffaloes*) and JF489134.1 for *Equus caballus* (*donkeys*). Bioedit program as used to align the retrieved sequences, where common conserved sequence among the seven species was used to design forward oligonucleotide primer (azz FOP). The non-conserved regions were used to design a specific-reverse for each species oligonucleotide reverse oligonucleotide primers (ROP; Table 2).

3. DNA extraction and evaluation of its adequacy for PCR amplification

Genomic DNA included mitochondrial DNA was extracted from muscle samples of goats, sheep, pigs, cattle, camels, buffaloes and donkeys using the Thermo Scientific Genejet Genomic DNA Purification Kit, *#k0721l* (www.thermoscientific.com/onebio) according to the manufacturer manual. Adequacy of extracted DNA for PCR amplification was evaluated by measuring its purity and concentration using NanoDrop 2000c, (Thermo Scientific, Wilmington, USA).

4. Conventional PCR amplification assays

PCR mix components and its performing steps were performed according to the manufacturer manual.

Amplimers were resolved by electrophoresis on 2% agarose gel (Promega, Giza, Egypt) run in TBE buffer (5.5 gm boric acid, 10.89 tris and 4ml EDTA (0.5 H) adjusted to PH 8, with the final volume is made up to 1L with distilled water). After being stained with 0.2 µg/ml ethidium bromide, the PCR product gel was densitometrically scanned and analyzed using Genus3, (Syngene, Berlin, Germany) and following the manufacturer's Quantity One Software Package.

In order to check the specificity of the oligonucleotide primers using the simplex PCR assay with raw meats, each set of oligonucleotide reverse primers was performed in simplex PCR with a non-investigated species to trace any case of cross-reaction. To confirm the specificity of the oligonucleotide reverse primers, the simplex PCR was carried out on the DNA samples extracted from one sample of each of the seven investigated species, and then from five samples representing each of these species.

The specificity of the oligonucleotide primers using the multiplex PCR with raw meats was tested analogous samples, mixed meats were used in a triplex PCR for meats of goats, sheep and pigs, a quadruplex PCR for meats of cattle, camels, buffaloes and donkeys and, finally, a heptuplex PCR to detect meats of goats, sheep, pigs, cattle, camels, buffaloes and donkeys. The three assays were performed in Techne Thermal Cycler TC-512 (Bibby Scientific Ltd, Staffordshire, United Kingdom) with the same steps and cycling parameters already given in Tables (3 and 4).

RESULTS AND DISCUSSION

Optimal primal sequence and appropriate primer concentration are essential for maximal specificity and efficiency of PCR. A poorly designed primer can result in little or no product due to non-fulfillment of a number of conditions among which (i) Primer length**:** In the present study (Table 2) oligonucleotide primers were at least 18 nucleotides in length to reduce the probabilities of confronting problems with a second hybridization site on the vector or insert. Oligonucleotide primers did not exceed 28 nucleotides as it is especially imperative to avoid 4 or more G's or C's in a row. Dieffenbach *et al*. (1995) indicated those oligonucleotide primers between 18 and 24 bp long can be very sequence specific if the PCR reaction annealing temperature is assigned within a few degrees of the melting temperature (T_m) of the primer (Table 2). The PCR reaction temperature used in the current work (Table 2) was set within degrees not exceeding 5°C of the primers T_m. (ii) Melting temperature: the melting temperatures for the primers used in the present work (Table 2) were in the range 55-60**°**C. Primers with lower T_m were sidestepped because of potential unclear results. Primers with higher T_m were also avoided because of potential PCR secondary annealing. (iii) GC-content: oligonucleotide primers had a

GC-content between 44 and 60 percent with the exception of pig primers (42 percent) for which the primer sequence was extended to 28 bp to keep the T_m above the recommended lower limit of 50°C. according to Rychlik *et al.* (1990), GC-content and T_m are strictly dependent on each other. (iv) 3'-End sequence: Care has been taken to ensure that oligonucleotide primers had higher GC-content on their 5' ends than on their 3' ends. This does not exclude the fact that a "G" or "C" is desirable on the 3' end. Kwok *et al.* (1990) stressed the importance of the 3' terminal position for the control of mis-priming. (v) Dimers: caution has also been taken in such a way that oligonucleotide primers do not contain sequences of nucleotides that would allow one primer to anneal to itself or to the other primer used in PCR reactions (primer dimer formation). This would result, according to Breslauer *et al.* (1986), in an unproductive priming event that reduces the overall signal obtained. (vi) specificity: oligonucleotide primers were chosen so that they have a unique sequence within the template DNA that is to be amplified. Oligonucleotide primers designed with a highly repetitive sequence were avoided as they result in a smear when amplifying DNA. (vii) Complementary oligonucleotide primer sequence: In the present work (Table 2) oligonucleotide primers were designed with absolutely no intra-primer homology belong 3 base-pairs. It was also avoided to permit inter-primer homology as this can interfere with hybridization. (viii) Suitability of primers to the size of the designed PCR products: As the specifics of the size of the desired PCR products often depend on the application, Dieffenbach *et al.* (1995) indicated that PCR products of 150-1000 bp are generally produced for the purpose of detecting DNA sequence. The species specific oligonucleotide primers in the present study were designed to amplify amplicons within this range (290-1000 bp) (Figs 1a, 1b and 2).

1. Adequacy of extracted DNA for species-specific conventional PCR amplification

The results given in Table (3) indicate that extracted DNA was adequate for species-specific conventional PCR amplification. The overall range was 12.35-46.29 (ng/µl), for concentration and 1.12-1.46 (OD_{260}/OD_{280} ratio), for purity. The goat extracted DNA showed the highest purity and concentration values (1.45 and 35.96, respectively) while the pig DNA showed the lowest values (1.31 and 12.56).

2. Simplex PCR specificity isolated from raw meats

PCR products amplified from meats of goats, sheep, pigs, cattle, camels, buffaloes and donkeys (Figs 1a and 1b) were single DNA fragments of 290, 370, 480, 580, 700, 800 and 1000 bp, respectively. The results indicate no case of cross-reaction was traced when a set of reverse oligonucleotide primers was performed in simplex PCR with a non-investigated species.

3. Multiplex PCR specificity of DNA isolated from raw meats

In a triplex PCR for meats of goats, sheep and pigs (Fig. 2, lane 9), a quadruplex PCR for meats of cattle, camels, buffaloes and donkeys (Fig. 2, lane 11) and a heptuplex PCR for meats of goats, sheep, pigs, cattle, camels, buffaloes and donkeys (Fig. 2, lane 13), products amplified, with species-specific oligonucleotide primers, retained the same specificity observed with simplex PCR (Fig. 2, lanes 1 through 7). A single band of target size was shown from one meat species without producing any fragment of non-specific amplification.

This work described a simplex and a multiplex polymerase chain reaction (PCR) assays for the accurate identification of two meat kinds forbidden in Islamic foods (pig and donkey meats) and five meat kinds commonly marketed in Egypt (goat, sheep, cattle, camel and buffalo meats). Meat samples from the seven investigated species were used for molecular analysis of each species as per standard method. *Cytochrome-b* gene was amplified by PCR using a common forward oligonucleotide primer. By mixing species specific reverse oligonucleotide primers in the appropriate ratio, DNA-fragments could be identified by only one multiplex PCR. PCR products were resolved by agarose gel electrophoresis and characteristic band pattern was observed for each species. The PCR products showed amplicons of 290, 370, 480, 580, 700, 800 and 1000 bp from goat, sheep, pig, cattle, camel, buffalo and donkey meats, respectively. The sequel of this study suggests that the method of detection used can be applied by quality control laboratories and inspection services to determine adulteration different kinds of meats and meat products.

The multiplex PCR (M-PCR) technique is a variant of PCR in which two or more DNA loci are synchronously amplified in the aforesaid reaction within a sole PCR mixture to produce amplicons of inconstant sizes specific to different DNA sequences. Henegariu *et al*. (1997) described critical parameters and step-by-step protocol of the M-PCR technique. M-PCR was used successfully in the present study and several previous works (e.g. Abd El-Razik *et al*., 2007; Bai *et al*., 2009; Sakalar and Abasiyanik, 2011). M-PCR proved to offer the following advantages: (i) PCR internal control: false negatives that pose potential problems in PCR are revealed in multiplex amplification since each amplicon allows internal control for the other amplified fragments. (ii) PCR efficiency of DNA template quality determination: with M-PCR technique degraded DNA templates show weaker signals for long bands than for short ones and for fall in amplification efficiency. (iii) PCR economic efficiency: by spotting multiple genes simultaneously, further information may be acquired from a single test run than would require more PCR reagents and preparation time. (iv) PCR rapidity: with M-PCR many meat products belonging to ruminants and non-ruminants can simultaneously analyzed in the same

reaction for example, in the present work 100 meat samples could have been analyzed in 25 reaction tubes at the same time by using seven primer sets belonging to goat, sheep, pig, cattle, camel, buffalo and donkey. The present study depicts (Fig. 2 lanes 9, 11 and 13) the consequences of an optimized M-PCR, which resulted in a single band of target from one species (goat, sheep, pig, cattle, camel, buffalo and donkey) and no fragment was produced by non-specific amplicon.

The results of the present study (Fig. 2) agreed with those of many works in obtaining different amplicon molecular length for animal species and, thus, capable to be used to differentiate between mixed-species meats lawfully or unlawfully produced. In few reports, however, cattle and buffalo (Jain *et al*., 2007), goat and sheep (Mafra *et al*., 2007), donkey and horse (Abdel-Rahman *et al*., 2009) showed the same amplicon molecular length. It was recommended to develop specific oligonucleotide primers for buffalo (Jain *et al*., 2007) and sheep (Mafra *et al*., 2007) and to use specific restriction enzyme following RFLP-PCR to distinguish between donkey and horse.

Finally, this study suggests an accurate, sensitive and rapid analytical technique for goat, sheep, pig, cattle, camel, buffalo and donkey meats and traceability identification based on PCR analysis of *Cytochrome-b* gene of mitochondrial DNA for enforcement of labeling regulations and ensuring that meats comply with religion regulations (*'halal*' authentication).

SUMMARY

The current study aimed to use the mitochondrial cytochrome b gene for authentication of meats from different animal sources, with special relevance to hala-related meat sources. In total, 35 raw meat samples were sampled, representing seven species, viz goats, sheep, pigs, cattle, camels, buffaloes and donkeys. Mitochondrial Cytochrome-b (mtCyt-b) gene sequences for the seven-investigated species were retrieved from GenBank database and aligned. Common universal forward oligonucleotide primer and specific-reverse primer for each species were designed. DNA was extracted, PCR was performed using conventional assay and amplicons were resolved using standard gel electrophoresis. M-PCR proved to offer the following advantages: (i) PCR internal control: false negatives that pose potential problems in PCR are revealed in multiplex amplification since each amplicon allows internal control for the other amplified fragments. (ii) PCR efficiency of DNA template quality determination: with M-PCR technique degraded DNA templates show weaker signals for long bands than for short ones and for fall in amplification efficiency. (iii) PCR economic efficiency: by spotting multiple genes simultaneously, further information may be acquired from a single test run than would require more PCR reagents and preparation time. (iv) PCR rapidity: with M-PCR many meat products belong-

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Table (1): Number and source of meat samples collected from the seven-investigated species in this study.

Species	Common name	Meat samples		
		Number	Source	Collection randomization adopted
Capra hircus	Goat	5	(1)	In 5 different days
Ovis aries	Sheep	5	(1)	In 5 different days
Sus scrofa	Pig	5	(2)	In 5 different districts
Bos taurus	Cattle	5	(1)	In 5 different days
Camelus dromedaries	Camel	5	(1)	In 5 different days
Bubalus bubalis	Buffalo	5	(1)	In 5 different days
Equus caballus	Donkey	5	(3)	In 5 different days

(1) El-Basateen abattoir, Cairo, Egypt. Local markets, Cairo, Egypt.
(2) Giza zoo, Giza, Egypt.

Table (2): Sequence, length, melting temperature and GC-content of the oligonucleotide primers designed for the seven-investigated species in this study.

Primer	Sequence	bp	Melting temperature (°C)	GC-content (%)
FOP	5' CCCAGCTCCATCAAACATCTCATC 3'	24	57.7	50.0
Goat-ROP	5' GACCTCGTCCGACATGTATG 3'	20	55.0	55.0
Sheep-ROP	5' GCTGTGGCTATTGTCGCAAATAGG 3'	24	58.4	50.0
Pig-ROP	5' CTGTTCCGATATAAGGGATAGCTGATAG 3'	28	56.0	42.9
Cattle-ROP	5' GTGGACTATGGCAATTGCTATGATG 3'	25	56.1	44.0
Camel-ROP	5' GTAGGGCTAGCATCAGTAGTAGTGC 3'	25	58.3	52.0
Buffalo-ROP	5' GCTGAATGGCCGGAACATCATAC 3'	23	58.4	52.2
Donkey-ROP	5' CTGGCTGTCCGCCGATTCAT 3'	20	60.2	60.0

CFOP: common forward oligonucleotide primer; ROP: reverse oligonucleotide primer.

Table (3): Ranges of concentration and purity of DNA of samples from the seven-investigated species (OD = Optical density).

Species common name	DNA	
	Concentration (ng/µl)	Purity (OD_{260}/OD_{280})
Goat	29.25-35.96	1.25-1.46
Sheep	14.26-17.88	1.18-1.42
Pig	12.35-12.56	1.12-1.31
Cattle	19.25-31.26	1.15-1.42
Camel	19.25-25.26	1.24-1.51
Buffalo	19.25-22.36	1.03-1.35
Donkey	39.25-46.29	1.12-1.44

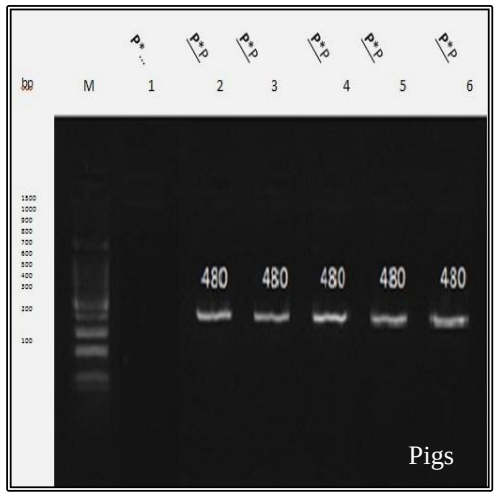

Fig. (1a): Agarose gel electrophoreses of simplex PCR products from five samples taken for meats of goats (G), sheep (S) and pigs (P). M: DNA ladder marker (one microliter contains 1500, 1000, 900, 800, 700, 600, 500, 400, 300, 200, 100 bp). The bold-faced letter refers to species-specific reverse oligonucleotide primer; the light-faced letter refers to species DNA. Lane 1 result when the set of reverse primers was performed in simplex PCR without template DNA. (negative control). Lanes 2 through 6 results when the set of reverse primers was performed in simplex PCR with a target species. * = with; the underlined (primer * DNA) refers to the same species.

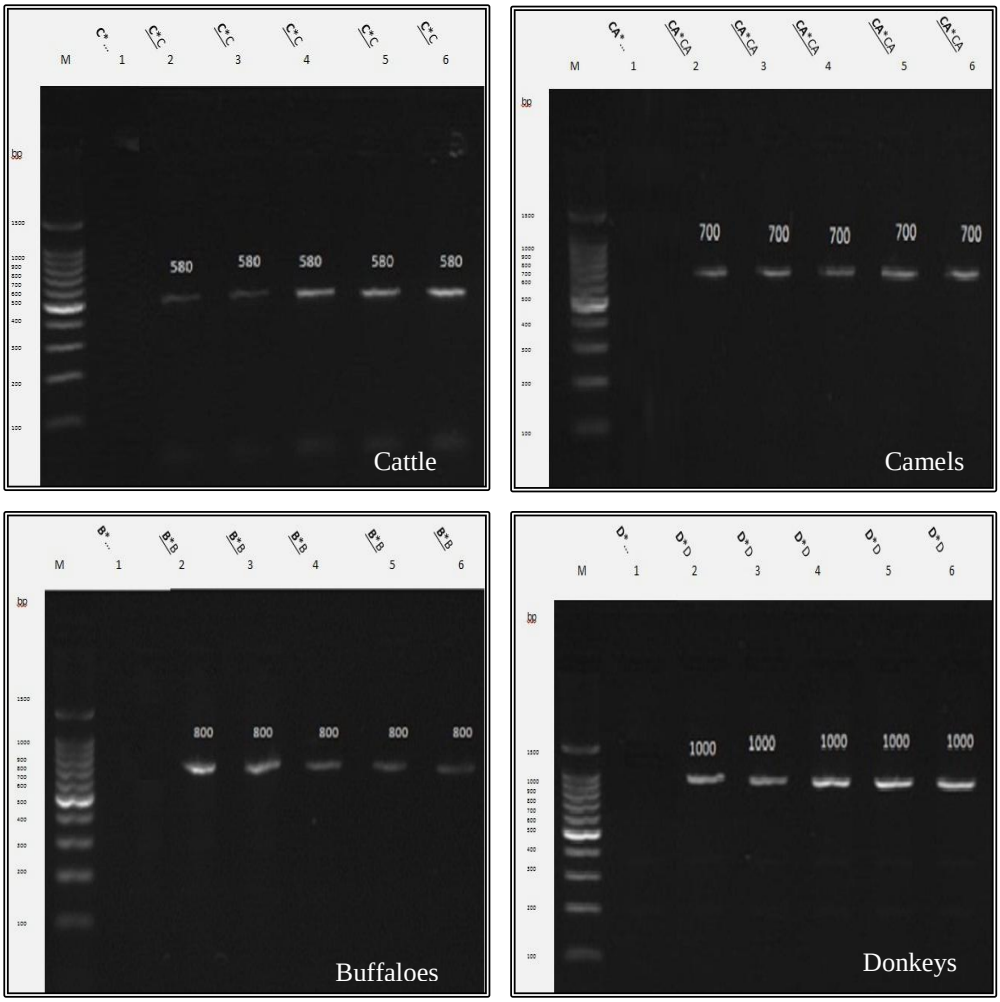

Fig. (1b): Agarose gel electrophoreses of simplex PCR products from five samples taken for meats of cattle (C), camels (CA), buffaloes (B) and donkeys (D). M: DNA ladder marker (one microliter contains 1500, 1000, 900, 800, 700, 600, 500, 400, 300, 200, 100 bp). The bold-faced letter refers to species-specific reverse oligonucleotide primer; the light-faced letter refers to species DNA. Lane 1 result when the set of reverse primers was performed in simplex PCR without template DNA... (negative control). Lanes 2 through 6 results when the set of reverse primers was performed in simplex PCR with a target species. * = with; the underlined (primer * DNA) refers to the same species.

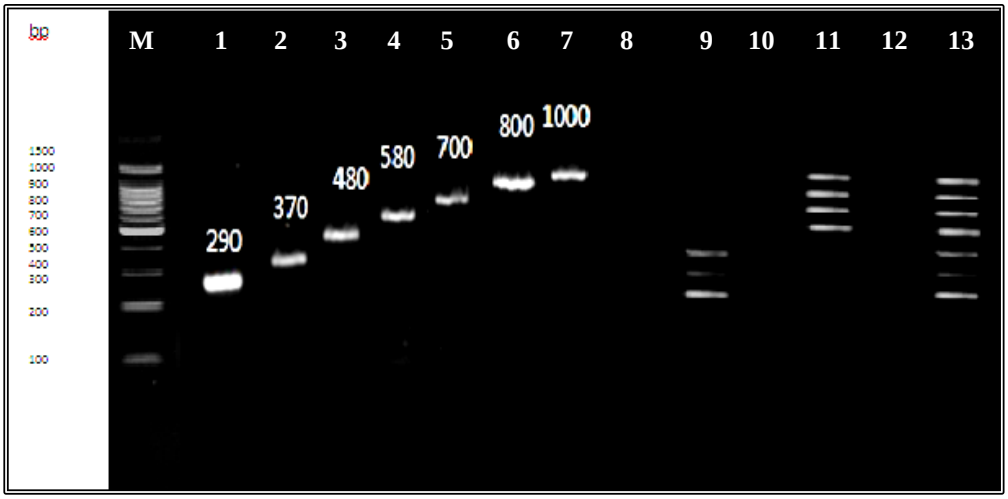

Fig. (2): Agarose gel electrophoresis of simplex, triplex, titraplex and heptuplex PCR products from meats of the seven species. M: DNA ladder marker (one microliter contains 1500, 1000, 900, 800, 700, 600, 500, 400, 300, 200, 100 bp). The bold-faced letter refers to species-specific reverse primer; the light-faced letter refers to species DNA. G: Goats; S: Sheep; P: Pigs; C: Cattle; CA: Camels; B: Buffaloes; D: Donkeys; * = with; the underlined (primer * DNA) refers to the same species or species mix. Lanes 8, 10 and 12 results when the set of reverse primers was performed in multiplex PCR without template DNA … (negative control). Lane 9: triplex; Goats, Sheep and pigs. Lane 11: quadruplex; Cattle, Camels, Buffaloes and Donkeys; Lane 13: heptuplex; Goats, Sheep, pigs, Cattle, Camels, Buffaloes and Donkeys.

1-**G***G 2- **S***S 3-**P***P 4- **C***C 5- **CA***CA
6- **B***B 7- **D***D 8- **(G+S+P)***… 9- **(G+S+P)***(G+S+P)
10- **(C+CA+B+D)***…. 11- **(C+CA+B+D)***(C+CA+B+D)
12- (**G+S+P+C+CA+B+D**)*…. 13- **(G+S+P+C+CA+B+D)***(G+S+P+C+CA+B+D)