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Pathogenicity of Different *Rhizobium radiobacter* (*Agrobacterium tumefaciens*) Isolates and Their Identification with Conventional Methods

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ABSTRACT

Rhizobium radiobacter is a significant causal agent that ranks among the top ten bacteria of molecular plant pathology in the world, has the largest range of hosts among plant pathogenic bacteria, and limits production and leads to economic losses in agriculture. The distinctive feature of the causal agent from other bacteria is the Ti plasmid, the extrachromosomal structure present in all virulent types. In this study, virulence of five *R. radiobacter* isolates (1A, 1B, 2A, 2B and RK 473) isolated from different rootstocks was tested in carrot slices, squash fruits, kalanchoe leaves, tomato and sunflower seedlings, and GF677, M9 and MM106 rootstocks, and hypersensitive response tests were conducted in tobacco plant. The isolates were diagnosed with biochemical and physiological tests by classical methods. All isolates formed tumors in carrot slices and squash fruits. 1A, 1B, 2A and 2B isolates formed tumors in the stem of GF677 peach rootstock, while it did not form any tumors on the stems of M9 and MM106 rootstocks. Tumor formation was observed in 1B isolate in the root application of GF677 peach rootstock, while no tumor formation was observed in other isolates. RK 473 isolate became pathogenic in M9 and MM106 apple rootstocks, while it was observed that the other isolates did not form any tumors. It was seen that none of the isolates became pathogenic in tomato and sunflower root and stem, and kalanchoe leaf applications. According to the virulence test results, 1B isolate was found out to be the most virulent isolate. Biochemical and physiological tests revealed the differences between isolates.

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Farklı *Rhizobium radiobacter* (*Agrobacterium tumefaciens*) İzolatlarının Patojeniteleri ve Konvensiyonel Yöntemlerle Tanımlanması

ÖZET

Rhizobium radiobacter, dünyada moleküler bitki patolojisinde ilk on bakteri içerisinde yer alan, bitki patojeni bakteriler içerisinde en geniş konukçu dizisine sahip olan, fidan yetiştiriciliğinde üretimi sınırlayan ve ekonomik kayıplara neden olan önemli bir hastalık etmenidir. Etmeni diğer bakterilerden ayırıcı özelliği tüm virülent türlerinde bulunan ekstrakromozomal yapı olan Ti plazmitidir. Bu çalışmada farklı fidanlardan izole edilmiş beş *R. radiobacter* izolatının (1A, 1B, 2A, 2B ve RK 473) virülanslıkları havuç dilimi, kabak meyvesi, kalonşe yaprağı, domates ve ayçiçeği fideleri, GF677, M9 ve MM106 anaçlarında test edilmiş ve tütün bitkisinde aşırı duyarlılık testi yapılmıştır. İzolatların biyokimyasal ve fizyolojik testler ile klasik yöntemlerle tanısı yapılmıştır. Tüm izolatlar havuç dilimi ve kabak meyvesinde ur oluşturmuştur. 1A, 1B, 2A ve 2B izolatları GF677 şeftali anacının gövdesinde ur oluştururken, M9 ve MM106 elma anaçlarının gövdesinde ur oluşturmamıştır. GF677 şeftali anacının kök uygulamasında 1B izolatında ur oluşumu gözlemlenirken diğer izolatlarda ur oluşumu gözlemlenememiştir. RK 473 izolatı M9 ve MM106 elma anaçlarında patojen olurken, diğer izolatların ur oluşturmadığı tespit edilmiştir. Tüm izolatların domates kök ve

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gövde, ayçiçeği gövde ve kalonşe yaprak uygulamalarında patojen olmadıkları görülmüştür. Virülanslık test sonucunda 1B izolatı en virülant izolat olarak belirlenmiştir. İzolatlar arasında biyokimyasal ve fizyolojik test sonuçlarında farklılıklar bulunmuştur.

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INTRODUCTION

Human nutrition of physiological and biological aspects with the discovery, it was revealed that nutrients such as carbohydrates, fats, proteins, vitamins, minerals and water in the structure of fruits are important for health (Yamankaradeniz, 1981).

In world, 65.220.334.00 hectares (ha) area 865.590.060 tons of fresh fruit was produced with China, India, Brazil and the USA placed on the top. Turkey ranks 5th with a fruit production capacity of 23.6 million tons, accounting for 2.68% of the world's total fruit production (FAO, 2019).

Türkiye has an important place in fruit growing as it has vast and fertile agricultural land and different environmental conditions, and is the homeland of various fruits produced in the world (Agaoglu et al., 1997).

In the world and Türkiye have an important position fruit growing sector is faced with several biotic/abiotic factors that may lead to loss of productivity and quality in terms of plant production throughout the process from seed stage to post-harvest stage. It is known that ~36% of the world's plant production are lost due to plant diseases, pest and weeds. 60-75% of these losses was grouped to arise from fungal and bacterial diseases, 10-15% from viral diseases, and 10% from other pathogens and environmental factors (Agrios, 1997). *Rhizobium radiobacter* (*Agrobacterium tumefaciens*) (Smith and Townsend) Conn., which leads to crown gall, is the most important causal agent resulting in great production and economic losses (Lippincott et al., 1981). Known to have spread to ~77 countries, the causal agent ranks among the top ten bacteria of molecular plant pathology in the world (EPPO, 2019). It has been reported that ~140 species of dicotyledons in more than 100 plant families [fruit trees (almond, apple, peach, cherry, apricot, pear, plum, walnut etc.), berries (raspberries, blueberries etc.), grapes and various ornamental plants (chrysanthemums, roses, poplars etc.)] were susceptible to crown gall and incurred great economic loss (Moore and Canfield, 1996; Marti et al., 1999; Collins, 2001; Gupta et al., 2010 Lacroix and Citovsky, 2016). The disease is tumor formation in consequence of uncontrolled growth, division and overmultiplication of parenchyma cells in the section of the host plant where the bacteria are present. In addition to tumor formation, it may lead to symptoms such as dwarfing and giving small and chlorotic leaves. These symptoms ultimately cause the plants to dry and die (De Cleene and De Ley, 1976; Saygılı et al., 2008).

R. radiobacter is a rod-shaped, gram-negative, motile, soil bacterium of *Rhizobiaceae* family, which has an approximate length of 1,5-3 µm and a diameter of 0.4-0.8 µm as well as 2-4 peritrichous flagella, is often found individually or in short chains, grows at an optimum temperature of 25-28ºC and loses its virulence at 37ºC, and does not form spores (Romanenko and Perepnikhatka, 1984; Collins, 2001).

The distinctive feature of *R. radiobacter* from other bacteria is that it is a plant pathogenic bacteria with circular Ti plasmid (Tumor Inducing Plasmid), which is present in all virulent types, known as extrachromosomal structure, and can transfer oncogenic DNA segment to susceptible plant cells and induce tumor formation (Roy, 2015).

Ti plasmid consists of one or more T-DNA (Transfer DNA) region(s) integrating into the plant genome, a vir region, a replication center, a conjugative transfer region, and regions containing genes required for opine catabolism. T-DNA contains the region to be integrated into the plant genome. In order that the causal agent can infect a plant, it should enter through a scratch or lenticel on the plant (Penyalver et al., 2000). The causal agent penetrating through plant tissues modifies the genetic structure of the plant, activates auxin-cytokinin group hormones and opine synthesis, and cause uncontrolled cell multiplication and formation of tumors in the roots (Zambryski, 1998; Kerr, 1991).

R. radiobacter is generally characterized and defined with morphological, biochemical, pathogenicity, antibiotic susceptibility and molecular methods. In this study, isolates were aimed to characterized by pathogenicity test, hypersensitivity test, biochemical and physiological tests.

MATERIALS and METHODS

Potential Pathogen and Bioagent Bacterial Isolates

Five different *R. radiobacter* isolates, which were

isolated from apple (RK 473), apricot (2A, 2B) and peach (1A, 1B) rootstocks were used.

Potential Pathogenic Bacteria Isolates Virulence Tests and Hypersensitive Responses

Carrot slices (*Daucus carota* L.) and squash fruits (*Cucumis pepo*), tomato (*Solanum lycopersicum* L.) and sunflower (*Helianthus annus* L.) seedlings, kalanchoe (*Kalonchoe daigremontiana* Raym.-Hamet and H. Perrier) leaves, and GF677 (*Prunus persica* x *P. amygdalus*) peach and M9 and MM106 (*Malus domestica* L.) apple rootstocks were used to test the pathogenicity of five potential pathogenic bacteria isolates, and to identify the most virulent isolate among them.

Virulence Test on Carrot Slices

The application was made according to Ryder et al. (1985). Then, 100 µL of each bacterial inoculum prepared according to Eastwell et al. (2006) was inoculated on the surface of carrot slices. 9 carrot slices were used in total, by repeating the application 3 times for each bacterium and using 3 carrot slices for each repetition. Sterile water was used for control. Petri dishes were evaluated according to expansion on the surface of carrot slices at the end of 30 days (1. no tumor formation, 2. tumor formation slightly expanding on the surface, 3. tumor formation starting around conducting tissue, 4. apparent tumor formation around conducting tissue and slightly expanding on the surface, 5. apparent tumor formation around conducting tissue and expanding on the surface, 6. dense tumor formation covering the whole surface), and the most virulent isolate was identified by analyzing the results with JMP 5.0.1 statistical analysis program (Limanska et al., 2015).

Virulence Test in Squash Fruits

The application was made according to Tolba and Soliman (2013). 12 wounds were inflicted on squash fruits with the help of a sterile toothpick, and 10 µL of bacterial inoculum prepared according to Eastwell et al. (2006) was applied on each wound. The squash fruits were placed in sterile transparent boxes covered with blotting paper, and kept waiting at ambient temperature in 16 hours light/8 hours dark. Sterile water was used for control. The size and number of tumors formed within 10-15 days after the inoculation were evaluated. Evaluations were made according to the earliest tumor formation and tumor diameters.

Virulence Test on Kalanchoe Leaves

Leaves of ~10-20 cm from young kalanchoe plants were used, and pathogenic bacteria isolates grown in NA for 48 hours were inoculated along opposite veins of the leaves with the help of a toothpick. Sterile water was used for control. 3 leaves were used for each pathogenic bacteria isolates, and 2 wound were inflicted on each leaf. Evaluations were made according to the presence/absence of tumors (Minnemayer et al., 1991).

Virulence Test on Tomato and Sunflower Seedlings and Rootstocks

Under greenhouse conditions, ~5 week old tomato and sunflower seedlings and ~30 cm peach (GF677) and apple (M9 and MM106) rootstocks were injured (Jaeger, 1974), and according to Eastwell et al. (2006) 100 µL of pathogenic bacteria inoculum was inoculated. Sterile water was used for control, and 3 wounds were opened on each of the 5 plants for each application. In seedlings presence/absence of tumor 8 weeks after inoculation; A scale was prepared according to the size of the tumors formed in the wounds of 6 months after the application of the rootstocks. According to the scale; 1. no change in the wound, 2. the wound is width of 0.5-1 cm, 3. the wound is width of 1-2 cm, 4. the wound is width of 2-3 cm, and 5. the wound is width of 3-5 cm evaluations were made (Jaeger, 1974). In addition, the roots of the seedlings and rootstocks were applied by immersing them in the pathogen solution. In root applications, evaluations were made according to the presence/absence of tumors.

Hypersensitive Response Test

A suspension with a density of 10^8 kob ml^{-1} was prepared in sterile dH_2O with pathogenic bacteria isolates grown for ~48 hours in NA medium. Each of the suspensions prepared were inoculated on the area between two side veins of tobacco plant (*N. tabacum* var. *Samsun*) with the help of a syringe. After 24-48 hours, formation of a transparent appearance in the area of bacteria inoculation was considered positive, while sterile dH_2O was used for negative control (Lelliot and Stead, 1987).

Identification of Potentially Pathogenic Bacterial Isolates by Conventional Methods

In order to support the diagnosis of potential pathogen isolates (1A, 1B, 2A, 2B, RK 473) with classical methods, biochemical and physiological tests were performed. These tests; growth at 23 and 35°C, 3-ketolactose production from lactose, growth on medium containing 2% NaCl, potassium hydroxide (KOH), acid purification in PDA+$CaCO_3$ medium, ferric ammonium citrate usage, alkaline formation from malonic, mucic and tartaric acid, acid production from melezitose, erythritol and sucrose, citrate usage, litmus milk reaction (Moore et al., 2001), and oxidase test (Kovacs, 1956) were made.

RESULTS and DISCUSSION

Potential Pathogen Bacteria Isolates Virulence Tests and Hypersensitivity Reaction Test Results

Pathogenic isolates virulence test results are given in Table 1. All isolates were pathogenic in carrot slices and squash fruits. 1A, 1B, 2A and 2B isolates formed tumors in the stem of GF677 rootstock, while they did not form any tumors in the stems of M9 and MM106 rootstocks. 1B isolate caused tumors but other isolates no tumor formation in the root application of GF677. RK 473 was only pathogenic isolate in M9 and MM106 stem applications, while other isolates no tumor formation. All of isolates were not pathogenic in tomato and sunflower root and stem, kalanchoe leaf.

According to virulence test results, 1B isolate was found out to be the most virulent isolate (Figure 1).

Figure 1. Tumors on carrot slices, squash fruit and GF677 rootstock caused by 1B isolate
Şekil 1. *1B patojen izolatının havuç dilimleri, kabak meyvesi ve GF677 anacında oluşturduğu urlar*

Many species in *Rhizobium* genus have a large range of hosts. However, it is recently known that some strains display very high host specifity based on Ti plasmid. (Knauf et al., 1982). Moreover, Owens and Cress (1985) showed that in consequence of tests conducted on different genotypes of soybeans, different *Rhizobium isolates* might display variations in tumor formation. They indicated that this might vary according to environmental factors and hormonal conditions, and these factors might play a key role in tumor formation. Anderson and Moore (1979) used 11 different plants to test the virulence of different isolates of *Agrobacterium* species, and observed that each isolate was pathogenic in different hosts. They indicated that these differences might arise from host susceptibility, virulence degree or interaction between the two. Deng and Nester (1998) also indicated that *R. radiobacter* isolate, which has a large range of hosts, could not cause tumor formation in all plants.

Theoretically, tumor formation may not occur due to reasons such as weak bacterial growth, bacteria's inability to survive at the scratch, lack of vir gene inducers or receptors required for bacterial connection in plants, or defective vir genes in T-DNA transfer. Moreover, it is known that tumor formation may not be induced due to failed T-DNA integration into the plant, absence of a full T-DNA gene set, or weak or excessive expression of T-DNA genes (Deng and Nester, 1998). In consequence of pathogenicity tests conducted in carrot slices, squash fruits, kalanchoe leaves, tomato and sunflower seedlings, and rootstocks in order to identify the most virulent among five different *R. radiobacter* isolates, which is the first objective of the study, it was observed that the isolates did not cause tumor formation in all test plants; in other words, there were differences in the pathogenicity of isolates (Table 1).The plants we used in pathogenicity tests are commonly used in *R. radiobacter* pathogenicity tests (Burr et al., 1995). In pathogenicity tests conducted for isolates, tumor formation was observed in some test plants and not observed in others, which showed that there were differences among hosts in terms of their pathogenicity. It was found out in the literature research that there were no rules indicating that an isolate might become pathogenic in all plants, and that there might be differences, as in the situations explained above. Among the plants used in pathogenicity tests, squash fruits (15 days) gave the clearest and quickest results, followed by carrot slices (30 days). Therefore, it was concluded that the use of squash fruits and carrot slices in pathogenicity tests were convenient (Figure 1). In the pathogenicity tests on 138 isolates of *Agrobacterium* species obtained from tissues with tumor, Yuzbasıoglu (2014) observed that some isolates did not become pathogenic in any plants except the one they were isolated from, some isolates became pathogenic in all indicator plants, and the

-Table 1. Virulence test results of potential pathogen isolates

Çizelge 2. Potansiyel patojen izolatlarının virülanslık test sonuçları

	Potantial Pathogen Bacteria Isolates Virulence Test Results (Potansiyel Patojen İzolatlarının Virülanslık Test Sonuçları)													
Test Plants *(Test Bitkileri)*	***RK473***		***1A***		***1B***		***2A***		***2B***		***Control***		**CV**	**LSD**
Carrot slices (*Havuç dilimleri*)	3.67±0.43	C	4.67±0.29	AB	4.89±0.24	A	4.00±0.48	C	4.22±0.29	BC	0.00±0.0	D	0.18	0.60
Squash fruits (Kabak meyveleri)	0.33±0.06	D	2.25±0.06	C	6.75±0.18	A	3.75±0.21	B	1.00±0.18	D	0.00±0.0	D	0.59	1.13
GF677 stem application (*GF677 gövde uygulama*)	0.00±0.0	D	1.86±0.72	B	2.73±1.29	A	1.33±0.47	AB	1.00±0.44	C	0.00±0.0	D	0.70	1.72
M9 stem application (*M9 gövde uygulama*)	2.47±1.02	A	1.00±0.0	B	1.00±0.0	B	1.00±0.0	B	1.00±0.0	B	1.00±0.0	B	0.34	0.31
MM106 stem application (*M9 gövde uygulama*)	2.39±0.96	A	1.00±0.0	B	1.00±0.0	B	1.00±0.0	B	1.00±0.0	B	1.00±0.0	B	0.41	0.36
GF677 root application (*GF677 Kök uygulama*)	-		-		+		-		-		-			
M9 root application (*M9 kök uygulama*)	-		-		-		-		-		-			
MM106 root application (*MM106 kök uygulama*)	-		-		-		-		-		-			
Sunflower seedling root and stem application (*Ayçiçeği fidesi kök ve gövde uygulama*)	-		-		-		-		-		-			
Tomato seedling root and stem application (*Domates fidesi kök ve gövde uygulama*)	-		-		-		-		-		-			
Kalonche leaves application (*Kalonşe yaprak uygulama*)	-		-		-		-		-		-			
Hypersensitive response test (*Aşırı duyarlılık reaksiyon test*)	+		+		+		+		+		+			

*****There is no statistically significant difference between the values expressed with similar letters on the same line ($p<0.01$), **+:** positive, **-:** negative

remaining isolates became pathogenic in one or two indicator plants. These results also support the conclusion that the pathogenic isolates used in the the study might not become pathogenic in all plants. Moreover, the necessity of using multiple plants instead of a single indicator plant in pathogenicity studies of *R. radiobacter* isolates was revealed.

Isolates (1A, 1B, 2A, 2B, RK 473) hypersensitive response test results are given in Figure 2. All isolates were showed slight yellowing and typical chlorosis after 48 hours. Kado and Crosa (1994) indicated that pathogenic R. radiobacter isolates mostly do not cause hypersensitive response, and only cause slight chlorosis in filtration areas.

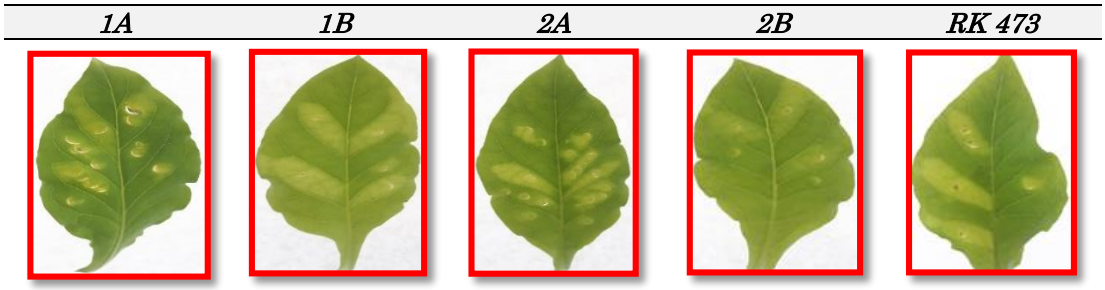

Figure 2. Hypersensitive reaction test result of potential pathogenic bacterial isolates in tobacco leaves
Şekil 2. *Potansiyel patojen bakteri izolatlarının tütün bitkisinde oluşturduğu aşırı duyarlılık reaksiyon test sonucu*

Potential Pathogen Bacterial Isolates Identification by Conventional Methods Results

Biochemical and physiological features have been the most useful features in the classification of tumorigenic *Rhizobium* species (Moore et al., 2001). Biochemical and physiological test results are given in Table 2.

According to biochemical and physiological test results, there are differences between *R. radiobacter* isolates.

It was found out that the isolates gave similar results growth in different temperature values (23 and 35°C), growth in medium containing 2% NaCl, KOH, acid purification in PDA+$CaCO_3$ medium, acid production from erythritol, litmus milk reaction, and oxidase tests (Table 2).

It was found out that they showed different reactions in the biochemical tests for 3-ketolactose production from lactose, ferric ammonium citrate usage, alkaline formation from malonic, mucic acid and tartaric acid, citrate usage, and acid production from melezitose and sucrose (Table 2).

While the test results for 3-ketolactose production from lactose were found out to be positive in relevant literature, test results for four of the isolates (1A, 1B, 2A ve 2B) used were found out to be negative (Table 2). The researchers also stated in their studies that 3-ketolactose production test might be negative (Lippincott and Lippincott, 1969; Kerr, 1969).

According to ferric ammonium citrate test usage, formation of a brick-colored layer on the tube was considered positive, and only RK 473 test result positive. (Table 2). Moore et al. (2001) said that isolates have might be differences at the level of 80%. In malonic and mucic acid tests, only RK 473 gave negative result and it was similar in literatüre research (Moore et al., 2001). In tartaric acid test, 1B and 2A isolates showed positive results while 1A, 2B and RK 473 gave negative results (Table 2).

RK 473 produced acid from melezitose and it was similar with Moore et al., (2001). All isolates except 2B produced acid from sucrose, this result was similar Moore et al. (2001) (Table 2).

According to citrate usage test results, only RK 473 isolate showed negative reaction (Table 2). Moore et al. (2001) said that *R. radiobacter* isolates may give different results.

Pulawska (2010) indicated that these differences were common among *Rhizobium* isolates forming tumors or fibrous roots. Similarly, Canik (2013) revealed that *R. vitis* isolates from vines showed different results in most of the biochemical and physiological tests.

Puławska et al. (2016) pointed out in the relevant study that the tests might give different results for strains belonging to the same species, and in some cases, same results for strains belonging to different taxons. As Pulawska explained, most important reason is that the bacterium generally uses different carbon sources in its metabolism (opine metabolism). Therefore, it was found out that a single definition system could not be sufficient for bacteria causing crown gall *R. radiobacter* (Puławska et al., 2016).

CONCLUSION and RECOMMENDATIONS

The study demonstrated that there may be differences the isolates which cause crown gall. It is important to make molecular identification in order to support their diagnosis with classical methods.

Table 1. Potential pathogen bacterial isolates biochemical and physiological test results
Çizelge 2. *Potansiyel patojen bakteri izolatlarının biyokimyasal ve fizyolojik test sonuçları*

Tests	***1A***	***1B***	***2A***	***2B***	***RK 473***
Growth on 35°C (*35°C gelişme*)	+	+	+	+	+
Growth on 23°C (*23°C gelişme*)	+	+	+	+	+
3-ketolactose production (*Laktozdan 3-ketolaktoz üretimi*)	-	-	-	-	+
Growth on medium 2% NaCl *(%2 NaCl besiyerinde gelişme*)	+	+	+	+	+
KOH test (*KOH testi*)	+	+	+	+	+
PDA+$CaCO_3$ acid purification (*PDA+$CaCO_3$ asit temizleme*)	-	-	-	-	-
Ferric ammonium citrate (*Demir amonyum sitrat kullanımı*)	-	-	-	-	+
Alkaline formation from malonic (*Malonik asitten alkali oluşturma*)	+	+	+	+	-
Alkaline formation from mucic (*Mucic asitten alkali oluşturma*)	+	+	+	+	-
Alkaline formation from tartaric (*Tartarik asitten alkali oluşturma*)	-	+	+	-	-
Acid production from melezitose (*Melezitozdan asit üretimi*)	-	-	-	-	+
Acid production from erythritol (*Eritritolden asit üretimi*)	-	-	-	-	-
Acid production from sucrose (*Sükrozdan asit üretimi*)	+	+	+	-	+
Citrate usage (*Sitrat kullanımı*)	+	+	+	+	-
Litmus milk reaction (*Litmus milk'te reaksiyon*)	Alk	Alk	Alk	Alk	Alk
Oxidase test (*Oksidaz testi*)	+	+	+	+	+

+: 80% and above positive, **-:** 80% and above negative, **V:** 21-79% between pozitive, **Alk:** alkaline

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Author's Contributions

The contribution of the authors is equal.

Statement of Conflict of Interest and Author's Contributions

Authors have declared no conflict of interest.

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