

SERO - AND MOLECULAR DIAGNOSIS FOR INCIDENCE OF *Maize yellow stripe virus* (MYSV) IN LOWER AND MIDDLE EGYPT

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ABSTRACT

Maize Yellow Stripe Virus (MYSV) is a leafhopper-borne *Tenuivirus* that has induced some epidemics on maize plants earlier in Egypt. Field samples from maize plantations were taken during the summer and Nily growing seasons of 2004 and 2005 to estimate the occurrence of MYSV on maize in ten governorates of Middle and Lower Egypt. Disease severity and percentage of MYSV incidence were recorded. Visual examination of MYSV symptoms, serodiagnosis using DAC-ELISA, and sometimes molecular tools (IC/RT-PCR and hybridization) were used to detect the disease occurrence in maize leaf samples. Generally, MYSV occurrence was higher in Middle than in Lower Egypt. Also, it was higher in Nily than in summer plantations. However, MYSV incidence in the summer season of 2004 was higher than in Nily season of the same year. The epidemiology and transmission of this virus by *Cicadulina chinai* is discussed.

Keywords: MYSV, Field Survey, Maize, DAC-ELISA, IC/RT-PCR, Hybridization.

INTRODUCTION

Maize is one of the important strategic cereal crops in Egypt. Most of the maize cultivated area is planted in spring (summer plantation), while the remaining area is planted in late summer (Nily plantation). In 2005 season, the total national production of maize reached about 6.825 million tons that resulted from an area of 1.927 million feddans, mostly of white grains with an average of 25.3 ard/fed. (8.5 tons/ha) (Anonymous, 2005). The endemic nature of some viral diseases of maize is one of the major factors responsible for low production in many countries (Thottappilly *et al.*, 1993). In a survey of maize virus and virus-like diseases in Egypt during 1981 and 1982, a new leafhopper-borne disease designated "maize yellow stripe" was discovered (Ammar *et al.*, 1984), and determined later to be caused by *Maize yellow stripe virus* (MYSV), which was proposed as new *Tenuivirus* member (Mahmoud, 2001; Ammar *et al.*, 2007). MYSV is now considered as one of the most important maize viruses which occasionally cause considerable losses in maize production as well as quality of the crop in Egypt (Thottappilly *et al.*, 1993; Aboul-Ata *et al.*, 1996). Three types of symptoms are associated with this disease: fine stripe, coarse stripe, and chlorotic stunt. Electron microscopy has revealed *tenuivirus*-like filaments in addition to helical

filamentous structures associated with infected maize leaves (Ammar *et al.*, 1989). MYSV-purified preparations contained fine filaments similar to those associated with *tenuiviruses* (Thouvenel *et al.*, 1996). Unlike *tenuiviruses* which are transmitted by delphacid planthoppers (Gingery, 1988), MYSV is transmitted in a persistent-propagative manner by the leafhopper *Cicadulina chinai* Ghauri, but not by *C.bipunctella zae* China, four other leafhopper and planthopper species, or mechanical means (Ammar *et al.*, 1989 & 2007). In addition to maize, MYSV infects sorghum, wheat, barley, oat and other graminaceous hosts (Sewify, 1994; Thouvenel *et al.*, 1996). During 1984 and 1985, incidence of MYSV was very high, particularly in late-sown maize plants in Giza and some other regions in Egypt (Ammar *et al.*, 1987&1990). Severe stunting and yellowing were observed in corn fields in Middle Egypt (Giza, Beni Suef, Fayoum, and Minia) in 1991, and several corn fields were removed because of severe-infection effect especially in Fayoum and Beni Suef (Anonymous, 1992; Aboul-Ata *et al.*, 1996). Severe stunting, leaf stripping, yellowing and curved apical stem in the Summer plantation of maize fields in Sohag, Quena, Beni Suef and Giza were also recorded in 2000 (Anonymous, 2001). The present work aimed at surveying maize fields for MYSV in several governorates, to determine the incidence and epidemiological behavior of MYSV, thus severe MYSV outbreaks may be explained and hopefully prevented. Advanced and traditional tools, *i.e.*, symptomatology, serological (DAC-ELISA), and molecular (IC/RT-PCR and hybridization) techniques were used for virus detection

MATERIALS AND METHODS

Field inspection and sampling

Incidence of MYSV was studied in maize fields initially using visual examination for external virus symptoms. Ten Governorates *i.e.* Middle Egypt (Giza, Fayoum, Beni Suef and Minia) and Lower Egypt (Qalyobia, Munofia, Sharkia, Gharbia, Ismailia and Kafr El-Sheikh) were visited during 2004 and 2005 growing seasons. Summer (July to August) and Nily (September to October) were the periods of field inspection. Surveyed fields were selected randomly in each Governorate. One to three fields were inspected in each location; and One to three locations were visited in each Governorate. Maize plants of different ages were examined in each visit. Percentage and severity degree of infection were estimated by visual examination for MYSV symptoms (yellow stripes parallel to leaf veins, leaves are smaller in size, plants are stunted and apical leaves are, sometimes, curved. Zigzag-line method previously described by (Smales *et al.*, 1996) was used for plant inspection. One leaf sample/plant was taken randomly from each of five plants. Leaf samples showing typical symptom of MYSV were collected and brought to the laboratory to confirm accuracy of visual inspection by other methods. All samples were stored at -20° until used for virus detection using ELISA or other methods. Percentage of infection was calculated by the following formula $(I/T \times 100)$ where I = number of infected plants, T = total number of tested plants. Degree of severity scale was determined according to (Aboul-Ata and Gordon, 1989) as follows: 1 = No symptoms observed, 2 =

Mild symptoms, 3 = Moderate symptoms, 4 = Severe symptoms and 5 = Very severe symptoms. Mean degree of severity was calculated by the following formula $(1 \times N1 + 2 \times N2 + 3 \times N3 + 4 \times N4 + 5 \times N5/T)$ where 1, 2, 3, 4, and 5 = different degrees of disease severity, N1, N2, N3, N4, N5 = Total number of plants those have specific degree of severity and T = Total number of plants examined.

Serodiagnosis

Serodiagnosis was used to confirm visual inspection and bioassay for MYSV test detection. Collected leaf samples were tested using direct antigen coating (DAC) ELISA, as described by (Clark and Adams, 1977; Hobbs *et al.*, 1987 and Mahmoud *et al.*, 1996) using MYSV-polyclonal antibodies kindly provided by Dr. Amal Mahmoud (Sadat city- Munofia Univ.). Samples were homogenized in 0.5:1 (wt/vol) coating buffer (0.05 M carbonate buffer, pH 9.6). Grinded samples were incubated overnight at 4°C. Micro plates were washed with PBS-Tween buffer 3 times. MYSV antiserum was used at a 1:8000 (vol/vol) in ELISA blocking buffer (Serum buffer) (13 m M NaCl, 1 m M KH₂PO₄, 8 m M Na HPO, 2.6 Mm KCl, 0.5 ml of Tween 20, 20.0 g of polyvinylpyrrolidone, 2.0g of ovalbumin, pH 7.4, distilled water to complete 1 liter). Treated plates were incubated at 37°C for 1hr. and washed as previously mentioned. Goat-antirabbit antibodies alkaline phosphatase conjugate was added at 1:10.000 in ELISA blocking buffer. Treated plates were incubated for 1hr. at 37°C and washed again. Substrate (p-nitrophenyl phosphate), in substrate buffer, was added and incubated for 1:30 - 2:0 hrs at 37°C. Volumes added at each step were 100µl/well in the micro titer plates. Positive control (insect-inoculated maize seedlings that showed MYSV symptoms and kept in insect-proof cages at the glasshouse) and negative control (Healthy maize seedling grown under insect proof cage) were used. ELISA-reader (EL_x 800 Universal Microplate Reader) was used for measuring test reaction at 405 nm wave length absorbance. Absorbance values more than twice the value of healthy control were considered positive.

Moleculardiagnosis

Moleculardiagnosis techniques aimed at confirming the serodiagnosed MYSV variability. Immuno-capture reverses transcriptional polymerase chain reaction (IC/RT-PCR) and dot blot hybridization were used.

IC/RT-PCR: One step IC/RT-PCR amplification of viral MYSV- RNA3 core region was used. Thermo-resistant polypropylene PCR tubes were coated with 50 µl MYSV-polyclonal antibodies (1/8000 dilution) and incubated for overnight at 4°C. Different MYSV-infected and non-infected samples were prepared in a dilution of 1/10 by grinding 0.3 gm of infected tissue in 3 ml sample extraction buffer, (4mM NaHPO₄, 1.8 mM KH₂PO₄, 2.7 mM KCl, 137 mM NaCl, PVP, pH 7.4). Antigen trapping on the inner surface of the PCR tubes occurred overnight at 4°C. The tubes were then washed twice with PBS-T, and once with deionized water. Extra care was taken to avoid cross-contamination between wells. The one step RT- PCR reaction was carried out in 50µl total volume of amplification mixture to reach the final concentrations of: 10mM Tris-HCl, pH 9.0, 50 mM KCl, 0.01% (W/V) gelatin,

4mM MgCl₂, 200 μM dNTPs, 0.1 μl each primer (Table 1), 2.5 units of the thermostable Taq DNA polymerase (Promega), 40 units ribonuclease inhibitor (Roche), 200 units of M-MLV reverse transcriptase (Promega) and incubating 45 min at 42°C. The amplification proceeded in the thermocycler (Uno) at 94 °C for 5 min, and through 35 cycles .Denature at 94 °C for 30 sec. and annealing 49°C for 30sec. and Extension at 72 °C for 2min, with a final step at 72 °C for 10 min.

Oligonucleotide primers: Newly specific non-degenerate primers were designed as shown in Table (1). The primer synthesized based on the nucleotide sequence of the ambisense MYSV-RNA3 according to Mahmoud, (2001). These primers amplify a DNA copy of approx. 720 base pairs. The virus sense primer F3 corresponds to nucleotides (nt) positions 79 to 103 of MYSV- RNA3 complete sequence, while complementary –sense primer R4 corresponds to nt positions 759 to 777. All used primers were synthesized at Operon Comp., USA.

Table (1): Oligonucleotide sequences of the used primers.

Primer	Primer size (mers)	Sequence	Prod.size
		5'-----3'	
F3	25	CTCAGCCATAGCCACATTACTCAGT	720bpx
R4	23	CTTTCAGGGTCAATTAGTTAGAG	

Dot blot hybridization: Half gram of MYSV-infected and non-infected leaf tissues were grinded in 2.5 ml of denatured solution containing [8X standard saline citrate (SSC) (1X SSC is 150 mM NaCl, 15mM Na acetate, pH 7.0) plus 10% formaldehyde]. The mixture was then heated to 60°C for 15 min and kept on ice. Ten μl of the supernatant of each extract were spotted onto pre-saturated nylon membrane (Boehringer Mannheim corp.) with 20X SSC. The membrane was then crosslinked by the UV irradiation for 2 min, followed by nucleic acid hybridization (Loebenstein *et al.*, 1997).

cDNA probe labeling: cDNA probe was labeled with digoxigenin, using PCR- Dig labeling technique according to Roche, Boehringer Mannheim corp., Indianapolis, IN, USA, protocol. The cDNA-PCR, which was previously produced in PCR reaction using forward (F3) and reverse primer (R4), was used as a template for PCR Dig Labeled dNTPs mixture.

Southern blot Hybridization: The methods of Southern (1975) and Sambrook *et al.* (1989) were followed. Electrophoresis products, of RT-PCR for MYSV using the two primers (F3 and R4) were gel electrophoresed and then were blotted on nylon membrane. Then, it was hybridized with nonradioactive cDNA- Dig labeled probe according to Boehringer Mannheim corp. instruction.

RESULTS

The results of field inspection for MYSV incidence is presented in Figs. (1 and 2) and Table (2). It was fluctuated from one year to another. It was higher in 2004 (10.7%) than in 2005 (2.7%). Generally, MYSV occurrence was prevalent in Middle Egypt (8.0%) than in Lower Egypt (6.1%). Disease severity was very mild (<1.5) in Upper Egypt and was very mild (<1.1). Also, MYSV incidence was higher in the Nily growing season (10.1%) than in the summer (2.1%) (Figs. 1 and 2). Results of serodiagnosis of the disease showed the same previous trend. Where it was higher in 2004 (16.8%) than in 2005 (13.8%). Also it was higher in Middle (19.5%) than in Lower Egypt (12.0%). Moreover, it was higher in the Nily season (19.4%) than in the summer growing season (13.5%). As regard to location, Munofia and Qalyobia (Lower Egypt) Governorates had the highest incidence level in the Nily season of both years based on visual examination (Figs. 1 and 2).

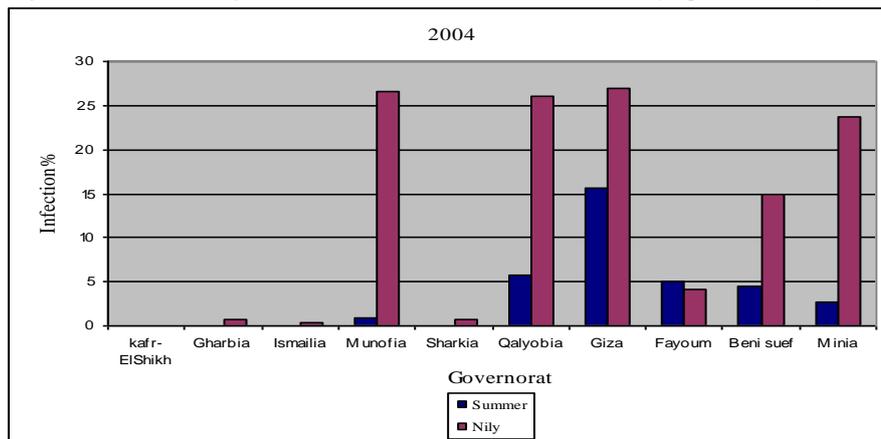


Fig. (1): Percentage of MYSV natural incidence during the summer and Nily seasons, 2004 in ten Egyptian governorates.

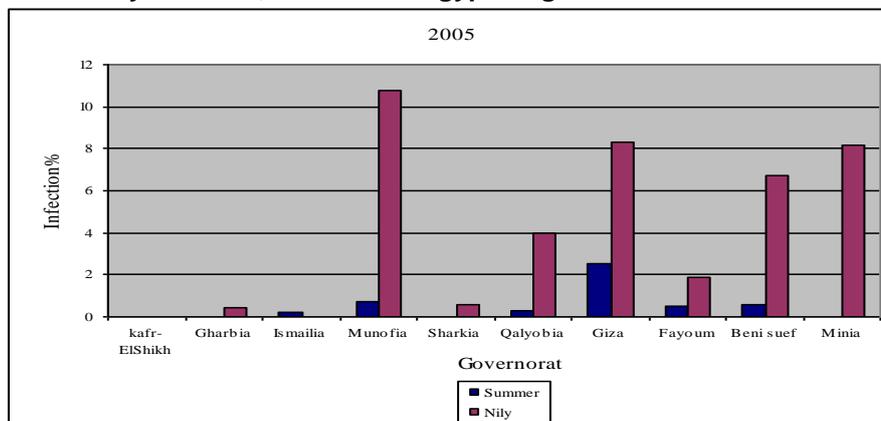


Fig. (2): Percentage of MYSV natural incidence during the summer and Nily seasons, 2005 in ten Egyptian governorates.

The previous trend has been confirmed using serological tests (DAC-ELISA) (Table 2). MYSV infection was 15 and 19.7% respectively in 2004 and it was 32.9 and 19% respectively in 2005. Ismailia was exception in the summer seasons in both years (36.0 and 22.2%, respectively). Giza, Minia and Beni Suef (Middle Egypt) had the highest MYSV incidence level during the Nily season of both years based on visual examination (Figs. 1 and 2). Also, MYSV infection has been confirmed based on serodiagnosis tests (Table 2). It was 26.7, 13.9 and 10.7%, respectively in 2004. However, it was 18.1, 28.6 and 36.4%, respectively in 2005. Summer growing season, had less MYSV infection based on visual examination but it was higher using DAC-ELISA as serological confirmation (Figs. 1, 2 and Table 2). Serological confirmation for the disease incidence had not the same trend. It was highest in Ismailia and Munofia (36 and 17.2% in 2004 and 22.2 and 15.8% in 2005, respectively). Giza, Fayoum and Beni Suef had the highest incidence (51.7, 21.7 and 29%, respectively in the summer of 2004). In 2005, these values decreased to (13.7, 13.8 and 4.5%, respectively). Data of MYSV incidence in of 2004 the summer season was higher than expected. These unexpected results were confirmed by the insect data during surveys, where the total number of viruliferous leafhopper *Cicadulina chinai* Ghauri, was higher in the summer (14.1%) than in the Nily season (2.3%) during 2004 (unpublished data). Moreover, percentage of viruliferous insect vector was higher (9.8%) in the Nily season than summer one (1.2%) during 2005 (unpublished data). This could explain differences in disease incidence data. On the other hand, the result of samples, which were selected (not random) with typical MYSV-like symptoms was (97.6%) tested positive in 2004, whereas (100%) in 2005. It means that visual examination for MYSV symptoms was 98.5% correct.

Table (2): Rate and percentage of the natural incidence of MYSV-infecting maize grown in Lower and Middle Egypt during summer and Nily seasons 2004, 2005 based on serological tests (DAC-ELISA).

	Season Location	2004		2005	
		Summer	Nily	Summer	Nily
Lower Egypt	Kafr- ElShikh	1/40 (2.5)*	1/33 (3.0)	0/26 (0.0)	1/84 (1.2)
	Gharbia	0/30 (0.0)	0/25 (0.0)	0/57 (0.0)	3/101 (3.0)
	Ismailia	9/25 (36.0)	10/56 (17.9)	18/81 (22.2)	0/68 (0.0)
	Munofia	5/29 (17.2)	12/80 (15.0)	6/38 (15.8)	47/143 (32.9)
	Sharkia	0/35 (0.0)	12/68 (18.8)	1/80 (1.3)	4/63 (6.4)
	Qalyobia	4/25 (16.0)	15/67 (19.7)	4/48 (8.3)	11/58 (19.0)
	Total	19/184 (10.3)	50/334 (15.0)	29/330 (8.8)	66/517 (12.8)
Middle Egypt	Giza	15/29 (51.7)	12/45 (26.7)	23/168 (13.7)	25/138 (18.1)
	Fayoum	13/60 (21.7)	5/65 (7.7)	9/65 (13.8)	25/128 (19.5)
	Beni-suef	20/69 (29.0)	3/28 (10.7)	3/66 (4.5)	32/88 (36.4)
	Minia	11/59 (18.6)	5/36 (13.9)	1/30 (3.3)	14/49 (28.6)
	Total	59/217 (27.2)	25/174 (14.4)	36/329 (10.9)	99/403 (24.6)
Total over all		78/401 (19.5)	75/508 (14.8)	65/659 (9.9)	165/920 (17.9)

* No. of infected /no. of tested plants, between brackets is a percentage.

More confirmation of MYSV occurrence, has been followed by using Immunocapture Reverse Transcriptional Polymerase Chain Reaction (IC/RT-PCR) to amplify viral RNA fragment in an artificially and naturally MYSV-infected maize plant samples (Fig. 3, lane 2). Specific primers (F3 and R4), were used to get intense DNA fragment at the expected size (approximately 720 bp of the RNA3). Extraction of total RNA from samples was unnecessary because the reverse transcriptase and PCR reaction mixtures had been applied directly to the virus particles in a single closed-tube.

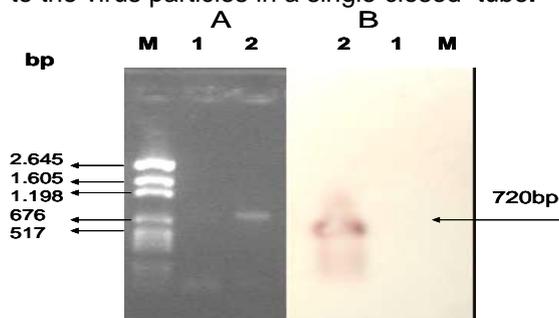


Fig. 3

Fig. (3): A:1% agarose gel electrophoresis analysis of IC/RT-PCR amplification products. Lane M: PGEM marker. lane1: No IC-RT-PCR products amplified from uninfected tissue. Lane 2: IC-RT-PCR products amplified from MYSV infected tissue. B: Southern blot hybridization using non-radioactive Dig labeled cDNA probe for detection of MYSV in infected tissue (Lane 2).

MYSV cDNA probe was clearly hybridized with the previous PCR products in Southern blot hybridization technique. Also, direct detection for MYSV-RNA in large amount of maize-infected tissues, dot blot hybridization on nylon membrane was used (Fig. 4). All tested samples and positive control (Fig.4, D9) were hybridized with the non-radioactive previously prepared Dig labeled-cDNA probe.

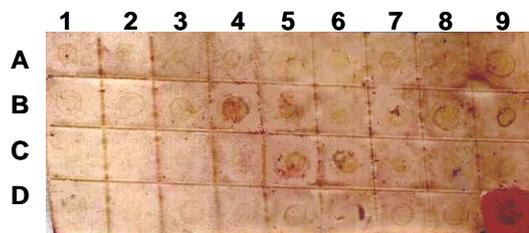


Fig. 4

Fig. (4):Dot blot hybridization on nitrocellulose membrane. The membrane hybridized with specific non- radioactive MYSV -cDNA -Dig labeled probe. From track A-1 to track D-8 are dot blot extract from field collected maize plants. Track D-9 is PCR and Track C-1, 2 are healthy samples.

DISCUSSION

Present study has used different methodologies *i.e.*, bioassay, sero- and molecular diagnosis for MYSV incidence determination which is more accurate than previous studies that was done by both Ammar *et al.*, (1987) and Aboul-Ata *et al.*, (1996). More general, MYSV incidence was higher in Nily season than in summer season which is normally stated by Ammar *et al.*, (1987) and Aboul-Ata *et al.*, (1996). Aboul-Ata *et al.*, (1996) indicated that MYSV was more prevalent in Middle Egypt (62.9%) than Lower (1.8%). In this study MYSV infection rate was not fluctuated that much between Lower (12.0%) and Middle Egypt (19.5%) (Table 2). Moreover, summer 2004 had higher incidence of MYSV (Table 2) in both Lower and Middle Egypt using sero-diagnosis and bioassay (is not shown). These findings, *i.e.* no much fluctuation of virus infection and high infection incidence in summer season, could indicate the virus ecological behavior. Not only that but also, Aboul-Ata *et al.*, (1996) proved that population density of viruliferous leafhopper *C chinai* vector was higher 2.2 times in population at 1991, where severe outbreak of MYSV was occurred, than 1992. Same situation, of viruliferous leafhopper vector, was stated in 2000 where one more severe outbreak of the virus was occurred than 2001 (Anonymous, 2001). This statement could also explain how virus ecology can be changed each 9 years. Severe outbreak of MYSV causes up to 83.9% yield loss (Aboul-Ata *et al.*, 1996).

Advantages, of using IC/RT-PCR for MYSV infection confirmation, was in high sensitivity, simplicity, more accuracy, low risk of contamination and the easy establishment of adequate conditions as well as direct application in a single closed-tube (Mehta *et al.*, 1997) which could help to identify changing virus ecology. It could be suggested that the virus, infection presented, is in an intermediate phase before having an expected severe outbreak in 2009. Noting that positive deviation of normal temperature (hot winter) is needed before summer growing season as it has been explained by Aboul-Ata, *et al.*, (1996).

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التشخيص السيرولوجي والجزئي لانتشار مرض التخطط الاصفر في الذرة في مناطق الوجة البحرى و مصر الوسطى.

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تم عمل حصر لانتشار مرض التخطط الاصفر في الذرة خلال الموسم الصيفي والموسم النيلي لعامى ٢٠٠٤، ٢٠٠٥ في عشرة محافظات، فى مصر. تم الكشف عن الفيروس في العينات المجمع من تلك المحافظات باستخدام الطرق السيرولوجية و الحيوية وكذلك طرق البيولوجيا الجزيئية باستعمال تفاعل انزيم البلمرة المتسلسل وتهجين الاحماض النووية. أظهرت النتائج أن مصر الوسطى كانت أعلى في انتشار المرض عن الوجة البحرى كما أظهرت النتائج أن إنتشار المرض في العروة النيلية كان أعلى منه في العروة الصيفي ولكن أظهرت الاختبارات السيرولوجية أنه في عام ٢٠٠٤ كان الإنتشار في العروة الصيفى أعلى منه في العروة النيلية . كانت نسبة الإصابة أعلى عام ٢٠٠٤ عن عام ٢٠٠٥ علي وجه العموم وقد استخدم الإستدلال العلمى لتفسير النتائج السابقة.