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Study of Secondary Endosymbionts of Whiteflies Collected from Cotton Growing Districts of Punjab, Pakistan

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ABSTRACT

Whitefly is the insect vector for cotton leaf curl disease. Like many arthropods, whitefly possess different primary and secondary endosymbionts depending on ecological zone and biotype. These endosymbionts play important role in several metabolic processes including reproduction. In this study, endosymbiont specific primers were used to check their presence in whiteflies. Each endosymbiont primer set amplify a PCR product of specific size and thus amplification of PCR product shows its presence in extracted DNA. Amplified DNA was checked using sodium channel gene-specific primers as internal control and the presence of endosymbiont was confirmed using 16S primers. We found multiple infections of endosymbionts in each district followed by confirmation through different molecular approaches. Our results confirmed the presence of multiple/diverse endosymbionts in whitefly samples collected from Punjab, Pakistan. The results would be helpful to correlate the occurrence of whitefly biotypes and disease intensity in different regions of Pakistan.

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Authors' Contribution
MYA, SM and IM conceived and designed the study. MYA executed the experimental work. MYA and SA analyzed the data. All authors contributed in preparation of manuscript.

Key words
Whitefly, Endosymbiosis, Cotton, PCR, CLCV.

INTRODUCTION

Whitefly a sucking insect is geographically distributed all over the world (Mound and Halsey, 1978). Whitefly is of economic importance more being a vector of viral disease than being a direct pest (Brown and Czosnek, 2002). Different whitefly biotypes are involved in geminiviral transmission (Haider *et al*., 2003). Host range for whitefly is very wide as they are found on citrus, cabbage, avocado, banana, cassava, coconut, capsicum, cotton, cauliflower, eggplant, guava, garlic, mango, legumes, mustard, peachy, onion, pepper, squash, radish, soybean, tobacco and tomato (Bellotti and Arias, 2001; Brown *et al*., 1989; Brown and Czosnek, 2002; Hoddle, 2006; Husain and Trehan, 1940; Kairo *et al*., 2001; Mani *et al*., 2004; Mellor and Anderson, 1995; Qazi and Khachatourians, 2005; Rose and DeBach, 1981; Sherf, 1986).

Whiteflies, aphids or mealybugs (phloem sucking insects) have primary and facultative relationship with endosymbionts of bacterial origin (Moran, 2001) like *Portiera* which is a primary endosymbiont (Baumann, 2005a). Besides primary endosymbionts, white fly harbors secondary endosymbionts namely *Hamiltonella* (Zchori-Fein and Brown, 2002), *Rickettsia* (Gottlieb *et al*., 2006), *Cardinium* (Zchori-Fein and Perlman, 2004), *Fritschea* (Everett *et al*., 2005), *Arsenophonus* (Baumann *et al*., 2004) and *Wolbachia* (Skaljac *et al*., 2010; Tram *et al*., 2003). Secondary endosymbionts are not localized to specific location, rather they are found throughout the host insect (Moran and Telang, 1998). Though these endosymbionts are not essential for survival rather they play important role in different metabolic processes of reproduction, development and viral transmission. *Wolbachia* plays role in reproductive manipulation in arthropods (Werren, 1997), whereas *Cardinium* plays its role in including cytoplasmic incompatibility, parthenogenesis and feminization (Zchori-Fein and Perlman, 2004). *Rickettsia* is found to be associated with improved fitness of whitefly by increasing its fertility, speedy development, more survival into adults and in increasing the population of females (Gottlieb *et al*., 2006; Himler *et al*., 2011). Apart from beneficial aspects sometimes *Rickettsia* population increases the susceptibility to insecticides and becomes harmful for its host (Kontsedalov *et al*., 2008). Moreover, in some cases susceptibility was higher for acetamiprid, thiamethoxam, sporimesifen and pyripoxen if co-infected with *Rickettsia-Arsenophonus* or *Rickettsia-Wolbachia* (Ghanim and Kontsedalov, 2009; Kontsedalov *et al*., 2008) in both B and Q biotypes (Chiel *et al*., 2007). *Hamiltonella* is host-dependent metabolic symbiont which depends on its host to fulfill its nutritional requirements (Degnan *et al*., 2009). *Arsenophonus* belongs to γ- Proteobacteria of phylum Proteobacteria is

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Table 1.- Primers used in this study, annealing temperature and amplified product size of these primers (Skaljac *et al*., 2010). Na channel is control to check quality of extracted DNA if it is amplifiable. 16S is control to check presence of bacteria in the template DNA.

Primer	Sequence	Annealing (˚C)	Product size (BP)
Hamiltonella	TGAGTAAAGTCTGGGAATCTGG AGTTCAAGACCGCAACCTC	62	700
Rickettsia	GCTCAGAACGAACGCTATC GAAGGAAAGCATCTCTGC	59	900
Fritschea	GATGCCTTGGCATTGATAGGCGATGAAGGA TGGCTCATCATGCAAAAGGCA	55	600
Cardinium	GCGGTGTAAAATGAGCGTG ACCTMTTCTTAACTCAAGCCT	59	500
Arsenophonus	CGTTTGATGAATTCATAGTCAAA GGTCCTCCAGTTAGTGTTACCCAAC	59	600
Wolbachia	CGG GGGAAAAATTTATTGCT AGCTGTAATACAGAAAGTAAA	55	650
16S	AGAGTTTGATCCTGGTCAGAACGAACGCT TACGGCTACCTTGTTACGACTTCACCCC	58	1508
Na^+ Channel	CGGTGAACTGGGTATCTTGG GGGAAACGAGACTTGGAATG	58	337

reported in many arthropods including aphid, louse fly, psyllids and whiteflies (Baumann, 2005b) which in whitefly plays role in transmission of virus by producing GroEL which interacts with coat protein and facilitates transmission (Rana *et al.*, 2012). It is found in Q biotype but not in B biotype whitefly in Israel (Chiel *et al.*, 2007).

Like *Wolbachia, Cardinium* has been reported that it causes cytoplasmic incompatibilities (Weeks *et al.*, 2003). In eukaryotes they produce some proteins which have potential to interfere cell cycle regulation this property makes it similar to Wolbachia (Penz *et al.*, 2012). *Fritschea* belongs to genus *Candidatus,* order Chlamydiales was for the first time reported in 2003 (Thao *et al.*, 2003) and is different from other endosymbionts in a manner that it is only documented in gut of whitefly. But it is absent in B biotype, lot is still to be discovered about its phenotype (Everett *et al.*, 2005).

MATERIALS AND METHODS

Whitefly samples were collected and stored in 80% ethanol from major cotton growing districts of Punjab, Pakistan. Whiteflies were placed in different tubes of 1.5ml, crushed (using 1ml pipette tips with pointed ends clogged for crushing by heating tips on flame) and homogenized in 80 µl of extraction buffer [100 µg/ml Proteinase K, 0.45% Triton, 0.45% Tween, 1 M Tris-HCl pH 8] (Ghanim *et al.*, 2007). This homogenate was then incubated for 1 hour at 55ºC and then for 10 minutes at 100ºC. This mixture was left for 5 minutes at room temperature before centrifugation. After centrifugation for 15 minutes at 13,000 rpm, the pellet was dried at 37ºC for 5 minutes after discarding the supernatant. Finally 30 µl double distilled (dd) H_2O was added to dried pellet to dissolve and the extracted genomic DNA was quantified on spectrophotometer (Smart Spec TM Plus, BioRad) at 260 nm absorbance.

Endosymbiont specific primers used in this study were same as reported by Skaljac *et al.* (2010).

PCR reaction of 50µL was made in 0.25µl tubes by adding 5 µl 10X *Taq* DNA polymerase buffer (Fermentas), 1.5 mM MgCl2, 5µL 2 mM dNTPs mix (dATP, dTTP, dCTP and dGTP), forward and reverse primers 0.5µM each, 1.25 units of *Taq* DNA Polymerase (Fermentas), 10 ng of template DNA and total reaction was made 50 µL by adding SDW. This reaction was run on thermal cycler (Eppendorf).

Agarose gel (1% w/v) with added ethidium bromide was made and put into gel apparatus tank containing 0.5 % TAE buffer. DNA mixed with 5X loading dye was loaded in each well and DNA ladder (Fermentas) as marker to estimate size of DNA was loaded in one of the well. The gel apparatus was given 100 volts electric current which move negatively charged DNA towards anode (positive terminal) and separates DNA according to its size. The DNA was checked under ultraviolet trans-illuminator system and picture was made using Stratagene Eagle Eye still video system.

Table II.- Samples were checked for Na Channel and 16S then used for PCR, description of co-infection of different endosymbionts in different regions of Punjab.

District	Na Channel (control)	*Hamiltonella*	*Rickettsia*	*Fritschea*	*Cardinium*	*Arsenophonus*	*Wolbachia*	16S
Sadiqabad	√	2/4	0/4	2/4	2/4	2/4	0/4	√
R Y Khan	√	1/4	0/4	1/4	2/4	4/4	0/4	√
Multan	√	3/4	1/4	3/4	0/4	0/4	1/4	√
Vehari	√	0/4	1/4	2/4	1/4	0/4	0/4	√
Khanewal	√	1/4	0/4	1/4	0/4	1/4	0/4	√
Bahawalpur	√	1/4	0/4	2/4	0/4	0/4	0/4	√

RESULTS

Whitefly extracted DNA was checked with Na Channel primers to confirm the quality of extracted DNA if it is amplifiable. The positive samples were then used for PCR using 16S primers to confirm the presence of endosymbionts in extracted DNA. The positive of this PCR too were then checked for endosymbiont specific primers. From each region four samples were used as template DNA while using endosymbiont specific primers. The results obtained are given in Table II.

The study showed co-infection of endosymbionts in whiteflies collected from some of the major cotton growing areas of Punjab, Pakistan. It is found that *Fritschea* is present in all samples, *Hamiltonella* endosymbiont is present in Sadiqabad, Rahim Yar Khan, Bahawalpur, Multan and Khanewal sample, but it was not found in Vehari. *Rickettsia* was only found in district Multan and Vehari samples but it was not present in other regions, *Cardinium* was present in Sadiqabad, Rahim Yar Khan and Vehari, *Arsenophonus* endosymbiont was amplified from Sadiqabad, Rahim Yar Khan and Khanewal. But *Wolbachia* was found only from Multan whiteflies sample.

Co-infection of endosymbionts was in almost all samples. In Sadiqabad and Rahim Yar Khan Hamiltonella, Arsenophonus, Fritschea, Cardinium were present, in Bahawalpur Hamiltonella and Fritschea were present, in Multan Arsenophonus and Cardinium were present, in Vehari Rickettsia, Fritschea, Cardinium were found but in Khanewal Hamiltonella, Arsenophonus and Fritschea were present.

DISCUSSION

In Pakistan there is different level of cotton leaf curl disease severity (Sattar *et al.*, 2013) the reason of which is well defined because different factors are involved in it. One possible reason is the presence of different biotypes of vector and the presence different of endosymbionts in whiteflies which ultimately produces different type of GroEL which have different level of affinity with different coat proteins of viruses. In China in 2003 Bing XL and his coworkers tested for five types of

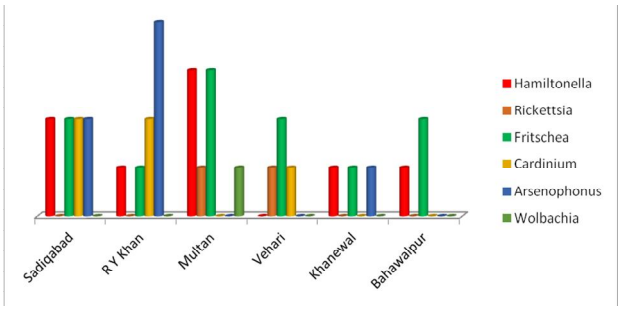

Fig. 1. District wise Co-infection of different endosymbionts.

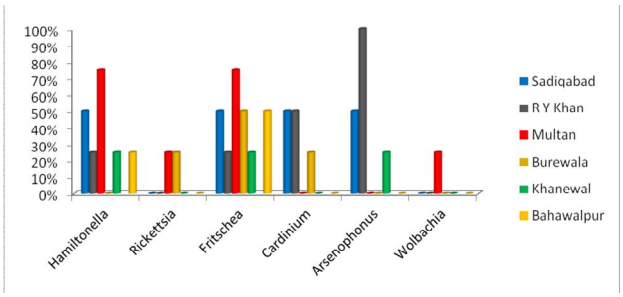

Fig. 2. Quantitative and relative presence of endosymbionts in each district.

secondary endosymbionts and found diversity and variation in their presence in whiteflies collected from different regions (Bing *et al.*, 2013). Using endosymbiont specific primers different endosymbionts were located in different regions of Brazil (Marubayashi *et al.*, 2014). In this study we have just found out the presence of different endosymbionts together in different cotton growing areas of Punjab and co-infection of different secondary endosymbionts in different combinations. *Wolbachia* is only present in Multan samples while Rickettsia is

present in Multan and its neighboring district Vehari but not present in other regions. Sadiqabad and Rahim Yar Khan with closely attached boundaries have same endosymbionts in whitefly. Bahawalpur which is a bridge between Multan and Rahim Yar Khan has two common endosymbionts that are *Fritschea* and *Hamiltonella*. Maximum of co-infection was seen for four endosymbionts in whiteflies from same region. Khanewal a neighboring district to Multan has two out of three endosymbionts common with Multan. Whitefly endosymbionts and their co-infection from Pakistan is reported for the first time which is novelty of this work.

CONCLUSIONS

The work presented in this article is first report for presence of different endosymbionts in different combination depending upon the location of area. In future we can correlate this occurrence with whitefly biotypes and disease severity in Punjab and then for Pakistan.

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Conflict of interest

Authors have no conflict of interest.

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