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STUDIES ON RECENT OUTBREAKS OF INFECTIOUS LARYNGOTRACHEITIS

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SUMMARY

Recently, increased incidence of outbreaks of infectious laryngotracheitis (ILT) was observed among chicken flocks in Egypt. These were associated with variable high mortalities. Investigation of 8 such outbreaks revealed that three nonvaccinated replacement layer pullets of 6-8 weeks of age were severely affected with mortality rates of 11-12% up to 40%, while in a 4th pullet flock of 24 weeks of age, which was comparatively milder and resulted in only 2 % mortality. On the other hand, in four commercial broiler flocks of 5-7 weeks of age with no history of previous vaccination, the disease varied in severity and mortalities (7.6 - 18.0%). Eight isolates were recovered from these outbreaks and were identified as those of ILTV. Pathogenicity tests for two representative isolates from pullets and broilers were carried out by inoculation of each intratracheally into susceptible chickens of the respective types (egg and meat-type), morbidity and mortality rates were used to calculate an intratracheal pathogenicity index used the same as that described for Newcastle disease virus, in addition to microscopic tracheal lesion scoring as criteria for judging their pathogenicity . Results indicated that both isolates were pathogenic like wild ILT field viruses.

Reversed virulence of modified live vaccine viruses was speculated under prevailing conditions of suboptimal management practices,

hygiene and biosecurity measures which help spread of infection between flocks, beside the role of latently infected carrier birds and other factors in the epizootiology of the disease, especially in nonvaccinated flocks, were discussed.

INTRODUCTION

Infectious laryngotracheitis (ILT) is an acute respiratory disease of chickens. The classical form of ILT is characterized by dyspnea, gasping, coughing and expectoration of bloody exudate (Whiteman and Bickford, 1989). The disease is caused by Gallid herpesvirus 1 (Roisman, 1982). Epizootic forms of ILT were often described in earlier years associated with mortalities of 20% or higher while the benign forms associated with low mortalities (0.1 - 2%) were the most regular feature of modern ILT in the developed intensive poultry industries of Europe, the USA or Australia (Hanson and Bagust, 1991). ILT had been recorded for the first time in Egypt since 1983 (Tantawi et al., 1983) and several commercial vaccines have been used to control the disease with success (Badr El-Din, 1985; Kamel, 1987). Recently , several outbreaks of ILT with variable mortalities appeared on farms, the majority of which were not vaccinated against the disease.

The objectives of this research were to investiagte the epizootiology of these outbreaks, and to isolate, identify and determine the pathogenicity of the isolates.

MATERIAL AND METHODS

History of examined chicken farms:

A total of 8 chicken farms naturally affected with ILT were investigated during 1997 - 1998. They included 4 replacement layer pullet farms and 4 commercial broiler farms located in 4 governorates. All affected farms had no history of vaccination against ILT except one farm (No.4). Further details on the history of examined farms are given elsewhere (Table 1).

Samples for ILTV isolation:

Postmortem examination was performed on a varible number of freshly dead birds which succumbed to the disease after onset of mortality on the examined farms. Gross lesions were recorded and birds with typical gross ILT lesions, mainly hemorrhagic tracheitis and conjunctivitis, were used for virus isolation. For this purpose tracheal exudate from 5-10 birds per farm were collected, pooled, labeled, and stored at - 20°C until further processed for virus isolation. In addition, 20 blood samples / flock were collected for serological examination by agar gel precipitation (AGP) test.

Fertile chicken eggs and chicks:

(a) Fertile chicken eggs and day-old commercial egg-type chicks originated from commercial

layer parent stock (Misr Hatchery Co., Giza locality), and day-old commercial broiler-type chicks from commercial broiler parent stock (Cairo Poultry Co., Giza locality). Both layer and broiler parent stock had previous history of routine prophylactic vaccination against ILT using live vaccines during the growing period. The eggs were used for virus titration, virus neutralization (VN), virus reisolation and antigen preparation for AGP test (AGPT) and the chicks for pathogencity test.

(b) Specific - pathogen - free (SPF) chicken eggs (USA) were supplied by Vet. Serum and Vaccine Res. Inst., Abbasia, Cairo. These eggs were used for primary virus isolation.

ILT viruses and antisera:

(a) Live commercial ILT vaccine (Intervet Inter. B. V. Boxmeer, Holland), obtained from local agency, was used for preparing AGPT - antigen.

(b) ILT - antiserum (Intervet Inter. B. V. Boxmeer, Hollond), supplied by Dr. A. K. Khafagy, Animal Health Res. Instit., Giza, was used in AGP and VN testes.

(c) Chicken anti - tenosynovitis serum (Intervet Inter. B. V. Boxmeer, Holland), supplied by Dr. A. A. Sami Ahmed, Fac. Of Vet. Med. Alexandria Univ., and chorioallantoic membranes (CAMs) of normal non - infected eggs were used as negative serum and antigen controls, in AGPT, respectively.

(d) Viruses used in pathogenicity tests: Two field isolates (No. 1 and No. 5 were used for these tests. They were passaged once on CAMs. Infected CAMs were harvested 120 hours postinoculation (PI), frozen at-20°C and thawed once, homogenized and suspended in aminollantoic fluid (AAF) of infected eggs (vol./vol.), and finally centrifuged at 1000 rpm for 10 minutes. The supernatant fluids were decanted and kept in small alliqutes at - 40°C until they were used. They were titrated in chick embryos and proved to have titers of $10^{4.8}$ and $10^{4.1}$ EID_{50} /0.1 ml for isolates No. 1 and No. 5, respectively. Each challenged bird was given a dose of 0.1 ml of the virus by micropipette instillation directly into the trachea (Anon., 1990). Details of the pathogenicity tests are summarized in the following table.

Virus isolation:

This was carried out by inoculation of 100% pooled tracheal exudate suspension in phosphate buffer saline (PBS, pH 7.2) containing 1000 IU of penicillin and 1 gm of streptomycin per ml into SPF - chicken embryos at 10-11 days of age by the CAM route in the usual way (Anon. 1990). Five embryonated eggs per sample were inoculated each with 0.1 ml of the suspension. Daily candling was performed and embryonic mortalities were recorded for 7 days post - inoculation. Mortalities during the first 24 hours

Experimental design for pathogenicity studies with two ILTV field isolates.

EXP. No	Birds			Experimental infection			Criteria adopted for evaluation of pathogenicity.			
	Type	No.	Age/ days	Group	Route	Dose	Virus	Serology	ITPI **	Virus re-isolation	Histopathology
1	Commercial pullets.	30	56	Infected.	Intratracheal	0.1 ml ($10^{4.8}$ EID_{50})	Infected CAM suspension of local isolate No. 1	1- Immune status pre-infection.	1- Clinical symptoms.	Pool of tracheal swabs in 50% glycerine -PBS at 4 days PI.	Lesion score:*** 1- Desquamation / deciliation. 2- Congestion. 3- Infiltration 4- Inclusion bodies. at 7 days PI.
		10	56	Controls.	Intratracheal	0.1 ml	* AAF of normal eggs	2- Sero conversion at 14 days PI.	2- Mortality for 8 days observation period PI.		
1	Broilers.	30	35	Infected.	Intratracheal	0.1 ml ($10^{4.1}$ EID_{50})	Infected CAM suspension of local isolate No. 5 * AAF of normal eggs				
		10	35	Controls.	Intratracheal	0.1 ml					

Exp. = experiment. No. = number.

* AAF = Aminoallantoic fluid.

** ITPI = Intratracheal pathogenicity index, determined by scoring each bird daily for 8 days PI after Hanson (1980) as follows:

***: Lesion Scoring after **Williams et al. (1992)**.

0- Normal.

1= Respiratory signs (coughing, gasping, conjunctivitis, and expectoration of blood).

2= dead; indices were determined by dividing the sum of scores by total number of observations.

*** Lesion scoring after Williams et al. (1992).

were considered non-specific and discarded, while those during 2-7 days PI and embryos that survived till the 7th day PI were examined for pock lesions on CAMs. The AAFs were checked for the presence of haemagglutinating (HA) agent against 10% chicken RBCs by the rapid HA test (Anon., 1990).

When visible pock lesions were absent, a second passage was done using 10% CAM suspension.

Virus neutralization test (VNT):

The alpha - procedure of VNT was adopted according to Hitchner et al. (1958). ILT antiserum diluted 1/10 (v/v) in PBS was mixed with an equal volume of 10- fold virus dilutions (10^1 - 10^{10}) in PBA and held at room temperature for 1 hour before inoculation onto CAM of 10-11 day old embryonated chicken eggs.

Agar gel medium:

It was prepared as described by Beard (1982) and consisted of 1.5 agarose, 8% NaCI, and 0.5 % phenol.

Virus titration (Hitchner et al., 1958):

Virus infectivity end - point of local field isolates No. 1 and No. 5 (used for experimental pathogenicity stduies) was determined by inoculation of ten - fold (10^1 - 10^{10}) virus dilutions in sterile PBS on CAMs of 10-11 day-old chick embryos using 5 embryos per dilution. The inoculum was 0.1 ml per egg. The eggs were candled daily for five days and deaths within the first 24 hours were considered non - specific. The CAMs of dead and survived embryos were examined for pock lesions. Calculation of 50% embryo infective dose (EID_{50}) was carried out according to Reed and Muench (1938).

Preparation of agar gel precipitation antigen:

ILTV antigen for AGPT was prepared from infected CAMs of embryonated chicken eggs 5 days after inoculation with 1/10 diluted ILTV vaccinal strain. The membranes with pock-like lesions were homogenized, then centrifuged at 3000 rpm for 15 min. and supernatant fluid was used as antigen. (Anon., 1990).

Agar gel precipitation test:

The test was used to demonstrate the presence of antibodies to ILT in chicken sera and for detection of ILTV antigen (s) in the CAMs of inoculated eggs as described by Beard (1982). Control positive and negative antigens or antisera were included in each plate, depending on the objective of the test (antibody or antigen detection). The plates were examined for specific preciptin line (s) after 24 hours incubation at 37°C and re-checked for final reading after 48 hours.

Histopathology:

Tracheas from experimentally infected birds (5

birds / group) that died 5-7 days PI and controls were fixed in 10% formalin solution and processed for paraffin sections in the usual way. Sections were stained with hematoxylin and eosin and evaluated according to the method described by Williams et al. (1992) for calculation of lesions index. Lesions were scored by 1 for the presence of each of the following four features: desquamation / deciliation, congestion, infiltration and inclusion bodies.

RESULTS

Characteristic features of ILT outbreaks:

During the period 1997 - 1998, 8 ILT outbreaks were investigated which occurred in 4 governorates and involved replacement layer pullets and commercial broiler chickens. All of these outbreaks occurred in ILT nonvaccinated flocks at 5-8 weeks of age except farm No. 4, which was affected at 24 weeks of age and had history of ILT vaccination via drinking water at 11 weeks (Table 1). They were characterized by mild to severe respiratory signs and lesions, and by mortality ranging between 2-40% in commercial replacement layer pullets and 7.6-18.0% in commercial broilers (Table 1).

The course of the disease was about 10-14 days. The clinical picture in all cases of commercial layer pullets (except farm No. 1) and broilers did not include the acute signs of ILT infection. The affected birds showed nasal discharge, lacrimation, coughing, wheezing and swollen eyes.

The main gross lesions were cattahral to haemorrhagic inflammation of the trachea, and conjunctivitis (Fig. 1); a blood-tinged mucous exudate or yellowish caseous material was present in some cases which at times formed plugs that occluded the tracheal lumen resulting in asphyxiation.

In farm No. 1, the affected birds showed severe dyspnea characterized by obvious extension of head and neck during inspiration, coughing and expectoration of blood which stained the walls and equipment of the house. The main gross lesion in this farm was haemorrhagic tracheitis, with clotted blood present in the tracheal lumen.

Serological examination of blood samples from affected farms by the AGPT (Table 1) revealed varied percentages (15-45%) of positive reactors in all flocks.

It is worthy noting that mangement and hygien were suboptimal in the majority of the investigated farms. In addition, most of the commercial pullet farms were multiple age operations with minimum biosecurity measures.

ILTV isolation and identifiaction:

ILTV was isolated from the 8 outbreaks following CAM inoculation of SPF - chicken

Table (1): History and seroconversion to ILTV infection of investigated chicken farms.

Bird type	Gode No.	Governorate	House capacity (number) / type of housing	Age / day at disease onset	Mortality *		Vaccination schedule	Seroconversion to ILT (AGPT) ^	
					Total	%		+ ve No./$ Exam. No	%
Commercial layers	1	Kalyoubia	10.000 / Floor	39	4.000	40.0	ND,IB, IBD.	9/20	45
	2	Kalyoubia	10.000 / Floor	55	7.100	11.0	ND,IB, IBD.	8/20	40
	3	Sharkia	20.000 / Cages	48	2.400	12.0	ND,IB, IBD.	9/20	45
	4	Giza	20.000 / Cages	168	400	2.0	ND,IB, IBD. ILT **	7/20	35
Commercial broilers	5	Gharbia	5.000 / Floor	31	840	16.4	ND, IB, IBD.	8/20	40
	6	Gharbia	5.000 / Floor	33	430	8.4	ND,IB,IBD.	5/20	25
	7	Gharbia	5.000 / Floor	29	900	18.0	ND,IB,IBD.	6/20	30
	8	Kafr El-Sheikh	5.000 / Floor	43	380	7.6	ND,IB,IBD.	3/20	15

* Recorded number for ILT mortality.
$ Vaccinated once at 77 day of age via drinking water with ILT live attenuated vaccine (Lohmann Animal Health Co.).
^ Against Known ILTV antigen.

embryos. Six isolates (No. 1 through 5 and 7) already gave pock-like lesions by the first passage, while the 2 remaining isolates (No. 6 and 8) by the second passge. Pock lesions appeared within 2-7 days PI and were characterized by having an opaque raised edge and gray central area of necrosis Fig. (2). Embryo deaths that occurred 2-7 days PI were characterized by stunted growth.

AGPT for ILTV antigen detection in CAMs of embryos that died during or were killed 7 days PI was positive with specific ILT antiserum. Rapid HA tests with AAF from dead and killed embryos proved negative.

All 8 isolates were completely neutralized by specific ILT-antiserum.

Response to experimental infection:

Clincal signs of ILTV experimental infection, including coughing, sneezing, conjunctivitis, gasping and deaths, began 3 days PI in both commercial pullets and broilers (Tables 2 & 3). Morbidity and mortality peaked (53.3%) at the 5th and 8th day PI, respectively, in infected

Table (2): Exp. 1 Cummulative clinical signs and mortality as well as calculated intratracheal pathogenicity index during 8 days observation of replacement layer pullets experimentally infected intratracheally with ILT field isolate No. 1 at 56 days of age.

Criteria	Day of observation								Total	Score	Total Scores	ITPI ❦
	1	2	3	4	5	6	7	8				
Cummulative signs	0	0	11	15	16	15	10	6	73	1	73	0.79
Cummulative mortality	0	0	2	5	8	13	15	16	59	2	118	
Normal	30	30	17	10	6	2	5	8	108	0	0	
Total No. of Birds	30	30	30	30	30	30	30	30	240	--	191	

❦ Intratracheal pathogenicity index determined after Hanson (1980) by dividing the sum of scores by total number of observations.

Table 3: Exp. 2 Cummulative clinical signs and mortality as well as calculated intratracheal pathogenicity index during 8 days observation of commercial broilers experimentally infected intratracheally with ILT field isolate No. 5 at 35 days of age.

Criteria	Day of observation								Total	Score	Total Score	ITPI Ψ
	1	2	3	4	5	6	7	8				
Cummulative signs	0	0	10	15	18	18	12	9	82	1	82	0.69
Cummulative mortality	0	0	1	4	6	9	11	11	42	2	84	
Normal	30	30	19	11	6	3	7	10	116	0	0	
Total No. of Birds	30	30	30	30	30	30	30	30	240	---	166	

Ψ Intratracheal pathogenicity index determined after Hanson (1980) by dividing the sum of scores by total number of observations.

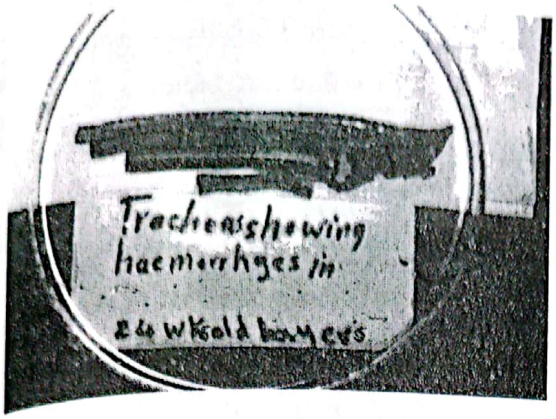

Fig. 1): Haemorrhagic tracheitis in 24 weeks-old commercial layer pullets naturally infected with ILT.

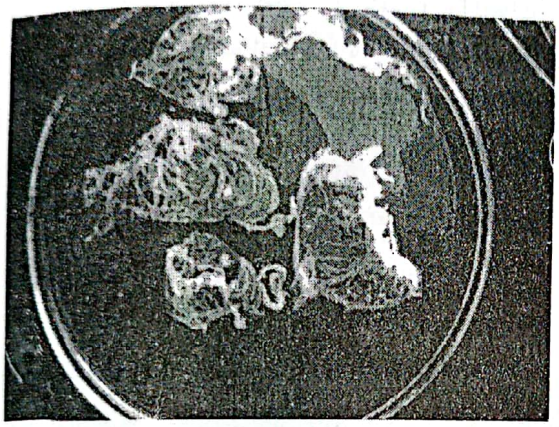

Fig. 2: Chorioallantoic membrane showing pock lesions 4 dys post-inoculation of 10 days old specific pathogen free eggs with field isolate No. 1.

Fig. 3: Trachea of 56-day-old replacement9ayer pullet experimentally infected with ILTV field isolate (No. 1), showing severe microscopic lesions (scored 4): detachment of the epithelium and inflammatory cell infiltration at 7 days postinoculation.

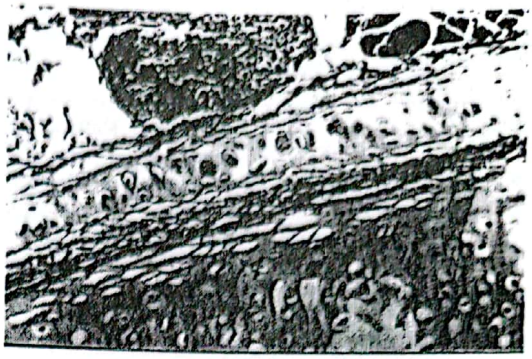

Fig. 4: Trachea of 35-day-old commercial broiler experimentally infected with ILTV field isolate (No. 5), showing seperation of epithelium and heterophils infiltration 7 days postinoculation.

commercial layer pullets, and at the 5th and 7th day PI, respectively (60% and 36.7%) in commercial broilers. Gross lesions in chickens of both types which died PI were confined to the trachea. Blood-tinged mucous to mucopurulent exudate in the lumen and severly congested tracheal mucosa were the predominant gross lesions. The histopathologic picture was characterized by degeneration, necrosis and desquamation of the tracheal epithelium, as well as haemorrhages and inflammatory cell infiltration (Figs. 3 & 4). Intranuclear inclusions could be detected in some epithelial cells. Tracheal lesion scoring after Williams et al. (1992) revealed maximum means of 4 for infected birds of both types (Table 4).

ILTV was recovered from tracheal swab pools at 4 days PI. Moreover, ILTV precipitating antibodies were detected in survivors at 14 days PI (7/14 in egg - type and 9/19 in broiler chickens) (Table 4).

Table (4): Results of experimental infection of replacement layer pullets and commercial broilers with ILT field isolates given intratracheally.

EXP. No	Birds			Antibody status at time of infection	Observations for 8 dpi				ITPI @	Seroconversion AGPT at 14 days PI		Microscopic tracheal lesions Ω	
					Morbidity		Mortality						
	Treatement	Age/ days	No.	No. AGPT + ves / Exam No.	Total No.	%	Total No.	%		+ ve No./ Exam. No.	%	+ ve No./ Exam. No	Mean lesion score
I	Infected pullets	56	30	0/30	16	53.3	16	53.3	0.79	7/14	50	5/5	4.0
	Control	56	10	0/10	0	0.0	0	0.0	0.0	0/10	0.0	0/5	0.0
II	Infected broilers	35	30	0/30	18	60.0	11	36.7	0.69	9/19	47.40	5/5	4.0
	Control	35	10	0/10	0	0.0	0	0.0	0.0	0/10	0.0	0/5	0.0

No. = Number. Exp. = Experiment. Exam.= examined. dpi = days postinfection.

@ Intratracheal pathogenicity index calculated after Hanson (1980).

Ω Microscopic lesions were scored after Williams et al. (1992).

DISCUSSION

The first report of exotic ILT infection in Egypt was recorded in the late 1982 by Tantawi et al. (1983). Since that time, prophylactic routine vaccination mainly of breeder and layer pullets with commercial modified live vaccines from different manufacturers has been the control policy of the disease which proved to be very effective. However during the last few years claims arose about increased incidence of the disease outbreaks in commercial layer pullets and broilers according to our observations. They were characterized by respiratory signs and gross lesions of varied severity typical of ILTV infection.

Investigation of 8 of such outbreaks in different leyer pullet and broiler flocks revealed that 3/4 layer pullet flocks experienced the disease at 6-8 weeks of age before the scheduled time of ILT vaccination. Mortality from the disease in these 3 flocks ranged between 11-12% up to 40% (farm No. 1), suggesting exposure to ILT viruses of variable pathogenicity. The fourth flock had been vaccinated at 11 weeks of age with live ILT vaccine (Lohmann Animal Health Co.) via drinking water and experinced the disease at 24 weeks of age with the lowest mortality rate of 2 %.

In the four commercial broiler flocks, which had no history of previous ILT vaccination, the

disease appeared at 5-7 weeks of age and also resulted in variable mortality of 7.6 - 18 %. Usually, broilers are not routinely vaccinated against ILT in Egypt, which allows field exposure to wild field ILTV and modified vaccinal strains as well, especially in some areas with high flock density. Suboptimal hygiene and management practices particularly common among small broiler units definitely contributed to field exposure to ILT virus. Secondary of concurrent infections together with management practices also contribute to the outcome of the disease, but the virulence of the invading field virus plays a major role.

Since bird-to-bird and flock-to-flock spread of ILT modified live vaccine viruses and their subsequent reversion to virulent forms is possible, especially those of chicknen embryo origin (Guy et al., 1991), it is highly probable that under prevailing conditions of suboptimal management, hygiene and biosecurity, which permit continuous contamination of the environment and spread of pathogens, the increased incidence of ILT outbreaks in non - vaccinated flocks may be attributed to virulence reversion. In this respect, the fact that some latently infected carrier birds remain in flocks naturally exposed to ILTV infection (Jordan, 1966) and in those vaccinated with live modified ILT virus vaccines as well (Bagust, 1986; Hughes et al., 1989, 1991), which perpetuate the infection to susceptible birds, should not be ignored.

ILTV could be isolated from all investigated outbreaks after 1-2 blind passages on CAM of SPF chicken embryos, and was identified by the AGPT and VNT against known ILT antiserum. Birds in affected flocks developed specific antibodies which could be detected by AGPT against reference ILT antigen.

Moreover, it was interesting to study the pathogenicity of the recovered isolates under laboratory conditions. Two isolates of different origin were chosen for this purpose; one from 39 days old commercial layer pullets (No. 1) that caused 40% mortality in the affected flock, and the other from 31 days old commercial broilers (No. 5) that caused 16.4% mortality. They were inoculated intratracheally into 8-weeks-old commercial pullets and 5-weeks-old commercial broilers, respectively, with respective virus doses of $10^{4.8}$and $10^{4.1}$ EID_{50}. Symptoms and lesions consistent with those of ILTV developed 3 days PI. Morbidity was comparable in both types of birds (53.3% and 60%, respectively), but mortality was markedly high and different (53.3 % and 36.7 %, respectively). An ITPI was calculated for 8 days observation period after Hanson (1980) and proved to be of value in distinguishing between the two isolates (ITPI = 0.79 and 0.69, respectively). Microscopic tracheal lesion scoring after Williams et al. (1992), however, revealed that both isolates have

a maximal mean index of 4.

Moreover, both types of birds showed seroconversion to the experimental infection as detected by the AGPT 14 days PI. These results suggest a high pathogenicity for the examined isolates which, however, did not accord with that of a vaccinal virus. It remains uncertain whether these isolates have their origin as wild field viruses or as wild field viruses or as virulence reversed modified vaccinal viruses. Restriction endonuclease - polymerase chain reaction (RE _ PCR) may help to solve this problem (Guy et al., 1990; Halina et al., 1996; Chang et al., 1997).

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