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A Multiplex PCR for Detection of *hlyA*, *papC*, and *traT* genes in multidrug resistance *Escherichia coli* isolated from pregnant women

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Abstract:

Urinary tract infection (UTI) in pregnancy is a common clinical problem which causes morbidity and, in a small minority of cases, renal damage and chronic renal failure. Urine samples from three hundred pregnant women were collected from Baghdad Teaching Hospital, were examined between January to June 2012 for the presence of bacteria. Their ages ranging from 15 to 50 years. Up to 32.6% (98) of women have a urinary tract infection (UTI). *Escherichia coli* was detected in 27.5% (27) of all the urine samples.

All isolates were screened for some virulence factors: α-haemolysin, adhesion factor type P fimbriae and serum resistance. Multiplex PCR systems were used to detect genes encoding α-hemolysin (*hly*A), adhesion P(*pap*C), and *tra*T associated with serum resistance. The results indicated that the occurrence of *papC* was detected in (18.5%), while *traT* was detected in (96.2%) of isolates on the other hand *hlyA* can't detected in any *E. coli* isolates. All the isolates were also studied for antibiotic susceptibility pattern using a disc diffusion method.

The rate of resistance of *E. coli* Amoxicillin (92.5%), Amoxicillin/Clavulanic acid (89%), Cefotazidime, Cefotaxime and Cefepime (78%), kanamycin (59%), levofloxacin (55.5%), Ciprofloxacin (52%) and showed high degree of sensitivity toward Cefoxitin and Imipenem (7% and 0%) respectively. Additionally,17 (62.9%) isolates were multidrug-resistant.

The finding of this study indicated that the *traT* widespread among pathogenic *E. coli* in Iraq. Furthermore it concludes that *E. coli* is one of the important multidrug resistance causative agents of urinary tract infection in young women especially during the state of pregnancy.

Key words: *pregnant women; UTI; E. coli; UPEC; Virulence genes; Multiplex PCR.*

الخلاصة:

يعد ألتهاب المسالك البولية عند الحمل مشكلة سريرية شائعة ، وفي حالات ضئيلة تسبب الضرر الكلوي والفشل الكلوي المزمن. تم جمع عينات البول من 300 أمراه حامل من مستشفى بغداد التعليمي، بين يناير حتى يونيو 2012 للتحري عن البكتريا حيث تراوحت أعمارهن بين 15 إلى 50 سنة.

اظهرت النتائج ما يصل الى 32.6٪ (98) من النساء مصابات بالتهابات المسالك البولية. تم الكشف عن *Escherichia coli* بنسبة 27.5٪ (27) من مجموع عينات الادرار كذلك تم تحري عن بعض عوامل الضراوة مثل ألفا هيمولايسين، عامل الالتصاق خمل نوع P ومقاومة المصل في جميع العزلات . استخدم نظام التفاعل التضاعفي لسلسة الدنا المتعدد للكشف عن جينات عوامل الضراوة (*hlyA*)، (*papC*) و(*traT*). حيث أشارت النتائج إلى أنه تم الكشف عن *traT* بنسبة (96.2٪) ، في حين تم الكشف عن *papC* بنسبة (18.5٪) من ناحية أخرى لم يتم الكشف عن *hlyA* في عزلات *E. coli* .

أختبرت حساسية العزلات للمضادات الحيوية باستخدام طريقة انتشار القرص. كان معدل مقاومة *E. coli* كالتالي Amoxicillin(92.5%), Amoxicillin/Clavulanicacid (89%), Cefotazidime, Cefotaxime, and Cefepime (78%), kanamycin (59%), Levofloxacin (55.5%), Ciprofloxacin(52%) وأظهرت درجة عالية من الحساسية تجاه Cefoxitin and Imipenem (7% and 0%) على التوالي . بالإضافة إلى ذلك اظهرت العزلات مقاومة متعددة والتي كانت نسبتها 62.9٪ (17). دلت نتائج هذه الدراسة على أن جين مقاومة المصل (*traT*) كان من الجينات الاوسع انتشارا بين عزلات *E. coli* المسببة للأمراض في العراق بالتالي من خلال هذه النتائج نستنتج ان بكتريا *E. coli* تعتبر واحدة من الانواع البكتيرية المهمة والمتعددة المقاومة المسببة لالتهابات المسالك البولية في النساء الشابات خصوصا خلال فترة الحمل.

Introduction:

Urinary tract infection (UTI) is one of the most common diseases encountered in clinical practice today. UTI is not only common but its range of clinical effect varies from asymptomatic bacteriuria to acute pyelonephritis[1]. UTI affects all age groups, but women are more susceptible than men, due to short urethra, absence of prostatic secretion, pregnancy and easy contamination of the urinary tract with faecal flora [2].

Pregnant women are at increased risk for UTI (starting in week 6 through week 24), because uterus sits directly on top of the bladder and displaces it. Shift in the position of the urinary tract and hormonal changes during pregnancy make it easier for bacteria to travel up the urethras to the kidneys. Additionally, the physiological increase in plasma volume during pregnancy decreases urine concentration and up to 70% pregnant women develop glycosuria, which encourages bacterial growth in the urine. The organism that causes UTIs during pregnancy are the same as those found in non-pregnant patients. *E. coli* accounts for 80-90% of infections [3].

The ability of *E. coli* to cause extra intestinal infections depends largely on several virulence factors, which help in the survival of *E. coli* under adverse conditions present in those sites. The virulence of individual strains in a given infection is determined by the presence and actual expression of the virulence genes present in them, and also by the environmental conditions in the host[4].Virulence factors (VFs) associated with Uropathogenic *E. coli* include adhesions (P fimbriae, type 1 fimbriae, S and F1C fimbriae, afimbrial adhesion), toxins (hemolysin, and cytotoxic necrotizing factor), serum resistance, siderophores (the aerobactin system) and polysaccharide coatings (group II capsules)[5].

Bacterial adherence to uroepithelial cells is an essential stage for the initiation and development of UTI. This process allows bacteria to resist the flushing action of the urine flow and bladder emptying, promoting bacterial persistence and activation of the host signaling pathways. UPEC strains are able to produce various types of adhesins necessary for the recognition and attaching to receptors along the urinary tract such as type 1 fimbriae, coded by the *fim* gene cluster; P fimbriae, coded by *pap* (pyelonephritis-associated pili) genes [6].

Production of toxins such as α-hemolysin causes tissue damage facilitating bacterial dissemination, releasing of host nutrients, and may also modulate host signaling pathways affecting several processes, including inflammatory responses, host cell survival, and cytoskeletal dynamics [7].

Also, the serum resistance, the ability to avoid the bactericidal activity of serum, is a characteristic important for pathogenesis enabling bacteria to persist in body fluids and internal organs. The outer membrane protein TraT play a role in the defense of bacteria against serum killing[8]. *E. coli* urovirulence factors can be analyzed by multiplex polymerase chain reaction (PCR) and are useful markers for the detection of uropathogenic *E. coli* [9].

Aim of this study:

The aim of the study was to determine the age groups in pregnant women are more susceptible to urinary tract infection and detect the virulence associated genes *hlyA*, *papC* and *traT* of *Escherichia coli* strains isolated from pregnant women by multiplex PCR technique.

Material and Methods:

Collection of Urine Samples:

Three hundred urine samples were collected during January to June 2012 from pregnant women (15 to 50 years) for isolation of *E. coli*, mid-stream urine

sample was taken for each pregnant women in sterilized test tubes[10].

Isolation and Identification of *Escherichia coli*:

For isolation of bacteria urine samples were first inoculated into MacConkey agar and incubated at 37°C for 24 h, after which a loopful was spread onto plates of Eosin Methylene Blue and further incubated at 37°C for 24 h. Isolates were further purified by picking discrete colonies (green metallic sheen) and sub culturing onto fresh plates of EMB and further incubating for 18 to 24 h at 37°C. After incubation, 1 to 2 discrete colonies were inoculated into the presumptive diagnostic medium sulfide - indole - motility medium (SIM) and incubated at 35°C for 24 h.Further characterization of isolates was carried out using the IMVIC test. Isolates that were indole positive, hydrogen sulfide negative, non-motile, as well as methyl red, Vogues - Proskauer and citrate utilization tests were identified as *E. coli*, fermentation of lactose, ability to produce indole, reaction on triple sugar iron (TSI) medium, hemolysis on blood agar, citrate utilization and motility of organism[11].The identifications were confirmed by the API 20E test system (Bio- Merieux).

Detection of Susceptibility to Antibacterial Agents:

Susceptibility of all the isolates to different antibiotics was determined by the disc diffusion method as recommended by the Clinical and Laboratory Standards Institute (CLSI)[12].

The antibiotic discs used in this study were Amoxicillin/Clavulanic acid (30mg; 20:10), Amoxicillin (30µg), Cefoxitin (30µg), Cefotaxime (30µg), Cefotazidime, (30µg), Ciprofloxacin (5µg), Cefepime (30µg), Imipenem (10µg), Levofloxacin (5µg), Kanamycin (30µg). Each antibiotic concentration was applied on the surface of Muller -Hinton agar plates inoculated with *E. coli* isolates and incubated at 37°C for 24 h. [13].

Hemagglutination and Expression of P fimbriae:

Colony adhesion factor (P fimbriae)**:** The expression of P fimbriae was determined by agglutinating human blood cells group A^+ in the presence of 0.1 M Tanic acid [14].

Adherence to Uroepithelial Cells:

The adherence capacity of the different bacterial isolates to uroepithelial cells was assayed as described by [15].

Haemolysin Production:

Plate hemolysis test was done for the detection of α-haemolysin produced by *E. coli*. The bacteria were inoculated onto 5% human blood agar and incubated over night at 37 °C.[16].

Preparation of Bacterial DNA:

The DNA to be amplified was extracted from whole organisms by boiling method. The bacteria were harvested from 1.5 ml of an overnight Luria - Bertani broth culture, suspended in sterile distilled water, and incubated at 95 ◦C for 10 min. Following centrifugation of the lysate, the supernatant was stored at -20 C◦ as a template DNA stock[17].

Multiplex PCR Amplification Procedure:

Detection of *hlyA* (α-hemolysin), *papC* (type P pili) and *traT* (serum resistance) genes was performed by amplifying the genes by multiplex PCR. The primers sequences were previously reported by [18,19] and obtained from Alpha DNA company (USA). Descriptions and sequences of the PCR primers used in this study are displayed in table-1.

Amplification was performed in a thermal cycler (Eppendorf, Germany) according to the methods described by [18].

The program, for *hly*A, *pap*C, and *tra*T genes the reactions mixtures included an initial denaturation at 94°C for 1 min consisted of 30 cycles of 94 ºC for one min, specific annealing temperature 63 C◦ for 30 seconds, and a final extension at 72 ºC for 90 seconds.

The detection of multiplex PCR products was performed on 0.8 to 1% agarose gels by electrophoresis and visualized under UV light.

Table-1: Sequence and molecular size of PCR products of *hlyA*, *papC* and *traT* genes.

Gene		Sequence of forward and reverse primer(5´- 3´)	Concentration Pmol/µl	Product bp
hlyA	F	AACAAGGATAAGCACTGTTCTGGCT	45632	1177
	R	ACCATATAAGCGGTCATTCCCGTCA	65380	
papC	F	GTGGCAGTATGAGTAATGACCGTTA	74929	200
	R	ATATCCTTTCTGCAGGGATGCAATA	56286	
traT	F	GGTGTGGTGCGATGAGCACAG	50167	290
	R	CACGGT TCAGCCATCCCTGAG	83607	

Results and Discussion:

Isolation of *Escherichia coli*:

A total number of 300 pregnant women of different trimester, their ages ranging from 15 to 50 years were examined in this study, from which 98 (32.6%) were found to contain heavy and appreciable bacterial growth (significant bacteriuria) while 202 (67.3%) had no appreciable bacterial growth (Table 2).

This is almost comparable to the prevalence of UTI reported in Saudi Arabia (35.2%)[20], but highest than study in Northwest Ethiopia (10.4%)[21].

Table-2: Incidence of UTIs in relation to age distributions of pregnant women.

Age group (years)	Number of specimen (%)	Number of positive (%)	Number of negative (%)
15-20	79 (26.3%)	25(25.5%)	54(26.7%)
21-25	112(37.3%)	38(38.7%)	74(36.6%)
26-30	55(18.3%)	15(15.3%)	40(19.8%)
31-35	37(12.3%)	15(15.3%)	22(10.9%)
36-40	15(5%)	3(3%)	12(5.9%)
41-45	1(0.3%)	1(1%)	0(0%)
46-50	1(0.3%)	1(1%)	0(0%)
Total	300	98(32.6%)	202(67.3%)

The incidence of UTIs in relation to age of the subjects shown higher percentage of pregnant women (38.7%) with UTIs within the age brackets of 21-25 years and the lowest percentage of (0.3%) was found in the age group of 41- 45 and 46 -50 years.

The highest number of bacterial isolates was obtained from pregnant women within the age brackets of 21-25 years; lower number of bacterial isolates was obtained from age groups 36-45 and 46-50 years as shown in Table 2. The study is in agreement with[21], but conflict with[22].

This variation may be due to low socio-economic status, sexual activity, washing genitals precious, post coitus, not voiding urine post coitus and washing genitals from back to front, and have observed as risk factors for UTI during pregnancy[23].

In this study, we found that more age ration group being susceptible to urinary tract infection in pregnant women groups is 23 years while under age ration group is 45.5, 48 years ranging in age between 15 to 50 years show in table-3.

Table-3: The relationship between age ration and positive number of UTI in pregnant women.

Age group (year)	Age ration	Number of positive (%)
15-20	17.5	25(25.5%)
21-25	23	38(38.7%)
26-30	28	15(15.3%)
31-35	33	15(15.3%)
36-40	38	3(3%)
41-45	45.5	1(1%)
46-50	48	1(1%)

Escherichia coli were the most predominant pathogen with over all isolation rates of 27(27.5%). The study is in agreement with[24] , but disagree with[20] .Also we can detect another types of bacteria causes UTI in pregnant woman but few number like *Klebsiella* spp., *Pseudomonas aeruginosa*, *Enterococcus* spp. and *Staphylococcus* spp.

E. coli is the most common microorganism in the vaginal and rectal area[25]. Because of anatomical and functional changes and difficulty of maintaining personal hygiene during pregnancy, may increase the risk of acquiring UTI from *E. coli*.

Although the bacteria *E. coli* isolates are common, but they are few, may be because of the occurrence of errors in the implant or due to poor growing conditions or incidence of urinary tract infections caused all kinds of bacterial, fungi or parasites.

Antimicrobial usceptibility Test:

Twenty seven strain of *E. coli* showed resistance to one or more antibiotics (Figure 1). Resistance was observed for all the antimicrobials tested: Amoxicillin (92.5%), Amoxicillin / Clavulanic acid (89%), Cefotazidime, Cefotaxime, and Cefepime (78%), kanamycin (59%), Levofloxacin (55.5%), Ciprofloxacin (52%) and showed high degree of sensitivity toward Cefoxitin and Imipenem (7% and 0%) respectively.

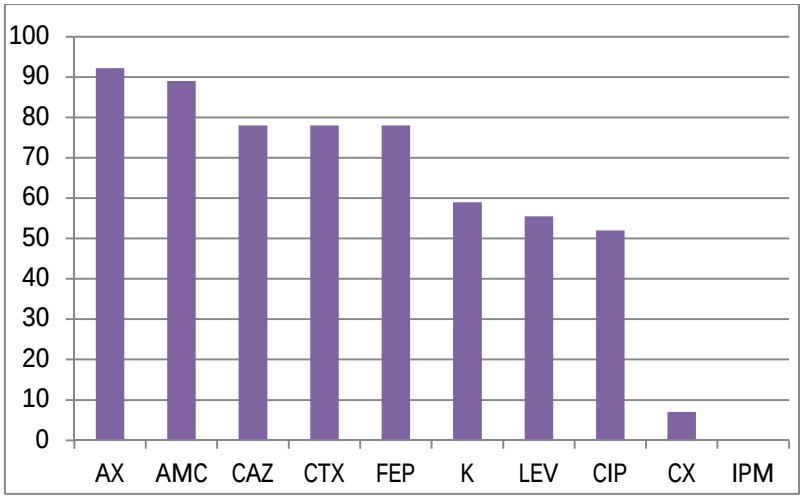

Figure-1: Percentage of isolates resistant to different antibiotics.

The most frequent antimicrobial resistance found was against Amoxicillin (92.5%), The study is agree with[26]. While *E. coli* showed high degree of sensitivity toward Cefoxitin (7%) that is agree with[18], and Imipenem (0%) that is agree with[27].

Multiple Drug Resistance Patterns of the Isolates:

The study showed table. 4 that the number and percentage of isolates of multidrug resistance is 17(62.9%) strains from a total of 27 *E. coli* isolated from urinary tract infections in pregnant women, while isolates with low resistance is 10(37%). Multi drug resistant bacteria are now a problem in hospitalized patients throughout the world.

The prevalence of MDR among clinical isolates vary greatly worldwide and in geographic areas and are rapidly changing over time[28].These results are in accordance with the results obtained in[21,37].

Tabl-4: Multi drug resistance pattern of bacterial isolates of pregnant women.

Groups	No. of antimicrobial which resisted by isolates	No. of the multidrug resistance isolates	Percentage of multidrug resistance
A	0-5	10	37%
B	6-10	17	62.9%

Phenotype and Genotype Detection of Virulence Factors in *Escherichia coli*:

The capacity of *E. coli* to produce many virulence factors contributes to its pathogenicity; these virulence factors enable some members of the normal flora to elicit an infection by overcoming the host defense mechanisms[29].

It was interesting to note that UPEC with virulence factors were significantly more prevalent. In this study we will examine a number of virulence factors produced by the *E. coli* isolated from UTI of pregnant women group. These virulence factors included the serum resistance, Hemagglutination (expression of P fimbriae) and α-hemolysin. The test results showed phenotypic as shown below (except serum resistance):

Adherence to uroepithelial cells:

Adherence of *E. coli* isolates to uroepithelial cells is used were showed that 27(100%) of the isolates presented adherence that mean number of bacteria adhered to uroepithelial cells ranged from 8 to 63. All isolates showed a mean number of bacteria/cells very close to that needed for a strain to be considered uropathogenic. The study is agree with[30] that showed the presence of adherence to uroepithelial cells is 65(100%).

Hemagglutination and expression of P fimbriae:

In the present study, 17(63%) UTI isolates of *E. coli* expression of P fimbriae by the bacterial strains was determined by agglutinating human blood cells in the presence of Tanic acid, show in table 5.The study is agree with[30] that showed the presence of expression of P fimbriae is 36(55%), but disagree with[33] which noticed the presence of expression of P fimbriae is 60(40%).

α-Hemolysin:

The strains of *E. coli* were tested for the ability to produce α-hemolysin that cause lysis of human erythrocytes.α-hemolysin production was detected by determining a zone of lysis around each colony on 5% human blood agar plates after overnight incubation. The result showed that out of 27 isolates of *E. coli* only 6(22.2%) show ability to hemolysis, because the hemolysis induction rapidly decreased, as previously described[31], and HlyA is a pore-forming toxin, at low concentration its binding not always lead to lysis[32], table-5.

The result is agree with[39] which showed that the number and percentage of hemolysin was 36(23.7 %), but disagree with[33] which showed that the number and percentage of hemolysin was 90(60%) of *E. coli* from UPEC.

Table - 5: The virulence factor and percentage of colony virulence factors pattern of *E. coli* clinical isolates.

Virulence factor	NO. of isolate (N27)	Percentage (N27)
Hemagglutination and expression of P fimbriae	17	63%
α-Hemolysin	6	22.2%

The PCR assay (Genetic diagnosis) was able to detect, *E. coli* virulence factor from a total of 27 *E. coli* isolates from pregnant women with UTI. These factors could be genotypically characterized by the use of multiplex PCR assay. For virulence factor encoding *traT*, *papC* and *hlyA* genes by using specific primers, multiplex PCR showed that the prevalence of virulent genes were present in 26 *tra*T (96.2%) , 5 *pap*C (18.5%) and *hly*A but it can't detected in any isolate of *E. coli* (Table 6). While *traT* gene was the most prevalent virulence factor detected, having occurred in 96.2% of strains.

The study also described serum resistance gene in *E. coli* strain at the genetic level which was, to our best of knowledge, the first study describing the serum resistance gene in UTI in Iraq. Serum resistance is the property by which the bacteria resist killing by normal human serum due to the lytic action of complement system.

Table-6: Distribution (number and percentage) of virulence factor genes in uropathogenic *E. coli* isolates from pregnant women of UTI.

Virulence factors (genes)	Number of positive strains	Percentage of positive strains (N27)
HlyA	0	0**%**
PapC	5	18.5**%**
traT	26	96.2**%**

In this study the *traT* gene associated with serum resistance, was the most prevalent, present in 26(96.2%) of *E. coli* strains. The study is agree with[18] that showed that the prevalence of *traT* gene is 76%.

Serum resistance (*traT*), may enable extraintestinal pathogenic *E. coli* to enter the primary infection sites (urinary tract or upper respiratory tract), spread to secondary internal organs, and survive in the blood stream, causing septicemia [38]. In addition, the prevalence of *traT*, significantly higher in *E. coli* isolates from urine, as previously reported in *E. coli* strains from urosepsis[19]. We assume that these virulence factors are more associated with urinary tract infection than with infections at other sites.

Since the binding to the host cells is a key factor for development of infection, the expression of different types of adhesins, with affinity to distinct specific receptors, confers advantages to pathogens[6].The pathogenesis of urinary tract infections depends on the *E. coli* skills to adhere, persist and multiply in the host[34]. The gene involved in bacteria adherence detected in our study was *papC*. The adhesion gene (*papC*) presented of 5(18.5%) of *E. coli* isolates. The study is agreed with [18] that showed the presence of *papC* gene is 25%.

In this study we note a few percent of *papC* gene, although this gene is one of the virulence genes commonly spread in cases of urinary tract infections. So this result is disagree with[30] where they showed a high rate of *papC* gene. This

gene has been associated with the presence of antibiotics resistance, but in this current study proved the opposite, where the results showed that there is no relationship between the presence *papC* gene and antibiotics resistance. Haemolysin production is associated with pathogenicity of *E. coli*, especially the more severe forms of infection [31]. In this study we did not find *hlyA* gene in any isolate of *E. coli*, although the presence of haemolysis in blood agar was observed in 6(22.2%) isolates.

This difference may be associated to silent expression or mutation in *hly* genes in *E. coli* [35], and prevalence of the *hlyA* gene vary according to the phylogenetic group, clinical conditions of host and geographical localization[36]. The study is agree with[18] that showed that the presence of *hlyA* gene is 5% of *E. coli* from UPEC.

The *papC* gene in the operon encoding P-pili was found in two isolate belonging to positive after amplification and the *traT* gene in the operon encoding serum resistance was found in seven isolate, while the *hlyA* gene in the operon encoding α-haemolysin was not found in any isolate belonging to positive after amplification and was shown in figure (2).

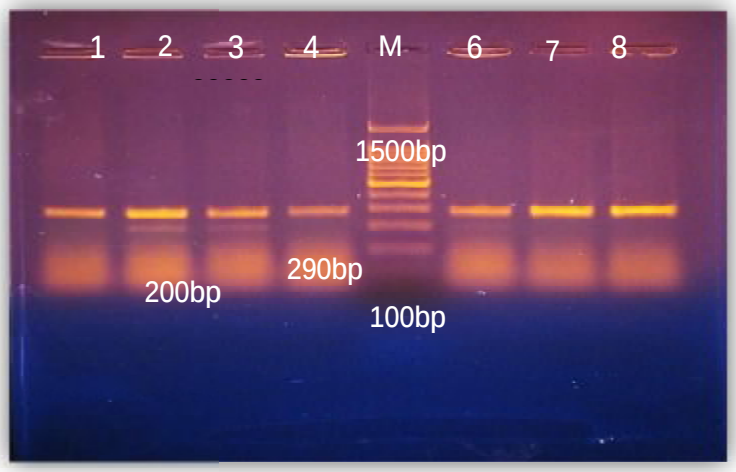

Figure-2: Multiplex PCR: Agarose gel electrophoresis (1% agarose,7 v/cm^2) and ethidium bromide staining to detect *hlyA*, *papC* and *traT* genes size products (bands 1177bp, 200bp and 290bp) respectively using template DNA prepared by boiling method. Lane 5 M, molecular size DNA ladder (100bp DNA Ladder); lanes 1-8, DNAs isolated from *E. coli* samples and only lanes 2,3,6 showed positive PCR bands *papC* ; lanes 1,4,6,7,8 negative; all lanes 1,2,3,4,6,7,8 showed positive PCR bands *traT*; and all lanes 1,2,3,4,6,7,8 negative PCR bands *hlyA*.

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