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BIOLOGICAL AND IMMUNOLOGICAL CHARACTERIZATION OF *ESCHERICHIA COLI* PILI ISOLATED FROM CASES OF URINARY TRACT INFECTION IN HORSES

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SUMMARY

The bacteriological examination of 126 urine samples from horses with urinary tract infection revealed the isolation of *E. coli* from 76 cases (60.3%); *Streptococcus* spp. 14 cases (11.1%); *Klebsiella* spp. 12 cases (9.5%); *Proteus* spp. 11 cases (8.7%); *Staphylosoccus* spp. 7 cases (5.6%) *Corynebacterium* 3 cases (2.3%) and 3 cases were bacteriologically negative (2.3%). The incidence of *E. coli* isolation from mares was higher (63.9%) than stallions (55.6%). Three *E. coli* serogroups were identified O:16, (26 isolates 20.6%), O:2, (20 isolates 15.9%), and O:6 (16 isolates 12.7%). The SDS-PAGE analysis of the purified pili preparations from each serogroup revealed one distinct protein band 20.1kDa for serogroup O:16 and O:6, whereas a 16.6kDa band for the serogroup O:2.

The hemagglutination activity of *E. coli* isolates to the different erythrocyte types was similar to their corresponding purified pili preparations. The effect of D-mannose on the hemagglutination of different erythrocytes revealed two types of hemagglutination, the mannose sensitive (pili type I) and the mannose resistant hemagglutination (pili type II).

In the in-vitro adherence test of *E. coli* isolates to urinary tract epithelial cells (UTEC), all *E. coli* isolates were highly adherent, especially the isolates of the serogroup O:16. In the adhesion inhibition test using the homologus antipili antisera, the number of *E. coli* adhered to UTEC was significantly ($P<0.01$) reduce, whereas the hetrologus antibodies could reduce the adhesion in case of isolates of the serogroup O:16 and O:6 only, while heterologus antipili antisera could not inhibit the adhesion of the isolates from the

serogroup O:2. Passively immunized white New Zealand rabbits with antipili hyperimmune serum that showed a high agglutination titer more than 1:640, tolerated the infection when challenged with homologus *E. coli* isolates. In heterologus challenges there was a cross protection between serogroup O:16 and O:6 and the adhesion was also significantly reduced ($P<0.01$), whereas rabbits passively immunized with hyperimmune serum against serogroup O:2 were not protected when challenged with heterologus serogroup O:16 or O:6.

INTRODUCTION

The attachement of bacterial pathogens to the uroendothelial cells has been considered as a potenial first virulence step in the induction of microbial pathogenesis in the urinary tract. Uropathogenic *E. coli* was incriminated as one of the most potential pathogens that cause pyelonephritis, cystitis and pyelitis in horses. Initial colonization of the *E. coli* might be ascending or descending in its pattern depending on the port of entery whether through the urethra or through the blood stream. (Krogfelt et al., 1991; Salyres and Whitt 1994 and Linhares et al., 1999).

Piliated strains in many bacterial models as *E. coli*, *Salmonella*, *Proteus* and *Moraxella* induced the infection whereas the nonpiliated isogenic variants were less pathogenic and in other occasions could not induce the infection. Pilus proteins were structured from repeating protein subunits called pilin which differed in their amino acid sequences, in their function as well as their receptors specificity. Variable factors affected the expression of *E. coli* pili in vivo as well as in vitro conditions as virulence of the bacterial strain involved, the surrounding niches pH, the presence of certain nutrients and the defence mechanisms of the host which could be humoral or cellular (Silverblatt et al., 1982; Naveh et al., 1984; Sexton and Reen 1992; Hamrick et al., 2000; Sauvonnet et al., 2000 and Greene et al., 2001).

Regarding the pili hemagglutination activity to various erythrocytes, two major types were identified. type I, in which hemagglutination was inhibited by mannose and was refered to as mannose sensitive (MS) and type II which was mannose resistant (MR). Protective humoral immunity was elicited against the immunogenic epitopes of the *E. coli* pili which might have shared antigenic determinant as well as a highly conserved epitopes, unique to each pilus type. These epitopes sometimes require cleavage of the pilus protein by certain chemicals to be exposed to elicit immune response (Korhonen et al., 1981; Greene et al., 2001).

The established recognition of the role played by the *E. coli* pili in the initiation of the uropathogenesis has prompted investigations of whether antibodies to pilus antigens might deter the

function of the pili by blocking the adherence and thereby cease the infection.

The objective of this study was to determine the role of *E. coli* in inducing pathogenesis in the urinary tract of horses as well as to elucidate virulence and immunogenic characteristics of their pilli which are considered a pivotal in the pathogenesis process.

MATERIALS AND METHODS

Specimens:

A total of 126 mid-stream urine samples were collected from horses (54 from stallions and 72 from mares) in governmental and private equine studs in Cairo and Giza governorates. The horses aged 3-10years and suffered generalized weakness, weight loss, inappetence, frequent painful urination and voiding of small quantities of cloudy urine.

Specimens were collected in screw caped sterile glass bottles and transferred to the laboratory in ice box for bacteriological examination.

Isolation and identification of bacterial isolates:

Urine samples were cultured directly onto blood agar plates containing 5% sheep blood and Mac Conkey agar. Plates were incubated at 37°C for 24-48 hrs. Colonies were identified morphologically, microscopically and biochemically according to Quinn et al., (1994) and by using the API 20E diagnostic streps kits (Biomereaux, France).

Serological identification of *E. coli* isolates:

E. coli isolates were identified serologically by slide agglutination test according to Edward and Ewing (1972) using polyvalent and monovalent *E. coli* antisera (Difco laboratories USA).

Preparation and purification of E. coli pili:

The different *E. coli* serotype isolates were grown onto veal heart infusion broth at 37°C for 24hrs. The bacteria were harvested by centrifugation at 6000xg for 15min at 4°C and resuspended in 0.01M Tris HCl. The bacterial suspension was placed on ice and then bacterial pili were sheared from cells by agitation in a homogenizer set at 70% speed for 5 minutes at 4°C. Cells were then centrifuged at 3000xg for 30 minutes and discarded. Supernatant which contained the pili was then dialyzed overnight at 4°C in 6000 to 8000 molecular weight restriction dialysis tubing against 2L of 0.05M Tris buffer (pH 9.5) with 0.01M sodium azide. Pili were then precipitated by adding ammonium sulphate to 50% saturation. The pellet which contained the pilli was resuspended in 0.01M Tris HCl and dialyzed in several changes of PBS pH 7.4 for 48hrs (Korhonen et al.,1982 and Greene et al., 2001). Then the protein content

of the pilli preparations was measured according to Lowry et al., (1952) technique.

The purity of the pili preparations was assessed by the sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Pilus preparation was diluted at 4-fold with sample buffer (0.25M Tris HCl, 0.5%SDS, 2.5% glycerol and 1µg/ml bromophenol blue) and incubated at 100°C for 4min. then loaded in a vertical slab of polyacrylamide using the discontinuous buffer system (Laemmli, 1970). Gels were stained with Coomassie blue or silver stain. Estimates of pilus protein molecular weights were determined by comparison of their migration distance with migration distances of low molecular weigt standards (Seleim, 1999).

Hemagglutination test:

All the *E. coli* isolates were prepared in 0.15M NaCl and adjusted photomitrically to 10% transmission at 620nm. Erythrocytes from humans (type A), horse and bovine and chicken were prepared in 3% suspention in PBS pH 7.4 after three successive washes, then equal volumes of RBCs suspension and the adjusted bacteria were mixed with or without D-mannose (25mg/ml). Parrallel to the previous test, the different pili preparations were tested to their hemagglutination ability of the different RBCs types (Hagberg et al., 1981).

In-vitro Adhesion of *E. coli* to urinary tract epithelial cells (UTEC):

UTEC were collected from freshly voided urine from healthy mares by centrifugation at 1000xg. The sedimented UTEC were washed and suspended in Hanks buffer saline (HBS) contained 10% foetal calf serum, then the cells were adjusted to 105cell/ml by hemocytometer. Equal volumes of the *E. coli* isolates adjusted to 1.0 optical density and stained with fluorescein isothiocyanate (FITC) were mixed with equal volume (0.5ml) of the adjusted UTEC and incubated at 37°C for 1hr. The non adherent bacteria were removed by washing in HBS. The adherent bacteria on 20 UTEC were counted under fluorescent microscope (Hagberg et al.,1981 and Seleim, 1997).

Antipili antisera preparation:

Antisera against each *E. coli* pili type was raised in New Zealand white rabbits. Purified pili protein was injected without adjuvant, 4 times, intravenously at one week intervals. The doses were 25, 50, 70 and 250µg. The rabbits were bled one week after the last injection. The titer of the antipili antibodies were measured as the highest dilution of antisera which agglutinated the piliated bacteria by the microagglutination test (Suwanichkul et al., 1988).

In-vitro Inhibition of adhesion:

E. coli isolates and the UTEC were prepared as mentioned in the adherence assay except that the bacteria were incubated with the antipili antiserum at 37°C for 30min before mixing with UTEC and the adherence inhibition was assesed as before (Svanborg-Eden et al., 1982).

Passive immuization of rabbits and challenge with *E. coli*:

Female New Zealand white rabbits aged 2 month old were passively immunized with 2ml rabbit antipili antiserum. Control rabbits received normal rabbit serum. All the rabbits were then challenged with the homologus and heterologus *E. coli* serogroup by intravenous injection of 0.5ml PBS pH7.4 containing $2.5x10^8$ *E. coli* cells. After 20 hours the rabbits were necropsied and the *E. coli* counts in kidney tissue were assessed (Bosch et al., 1979).

The data of *E. coli* adhesion and adhesion inhibition were subjected to T test analysis (Snedecor and Cochran, 1967).

RESULTS

The bacteriological examination of the midstream urine samples from the clinical cases revealed the isolation of *E. coli* from 76 cases (60.3%) other bacteria were also isolated but in lesser frequencies as *Streptococcus* spp. 14 cases (11.1%); *Klebsiella* spp. 12 cases (9.5%); *Proteus* spp. 11 cases (8.7%); *Staphylosoccus* spp. 7 cases (5.6%); *Corynebacterium* 3 cases (2.3%) and another 3 cases were bacteriologically negative (2.3%).

The incidence of isolation of *E. coli* from mares urine was higher (63.9%) than stallions (55.6%). These isolates belonged to three serogroups O:16 (20.6%), O:2 (15.9%), and O:6 (12.7%) as well as 15 *E. coli* isolates were untypable (11.1%) (Table1).

The SDS-PAGE analysis of the pili preparations revealed one distinct protein band 20.1kDa specific for the serogroup O:16 and O:6, whereas a 16.6kDa band for the serogroup O:2 (Figure1). The hemagglutination activity of *E. coli* isolates to the different erythrocyte types was similar and their corresponding purified pili preparations were similar. The effect of D-mannose on the hemagglutination of different erythrocytes revealed two types of hemagglutination, the mannose sensitive (pili type I) and the mannose resistant hemagglutination (pili type II) (Table2).

Table 1: Different *E. coli* serogroups isolated from horses had urinary tract infection.

Bacterial isolates	Stallion (n*=54)	Mares (n =72)	Total (n=126)
	No of isolates, Incidence rate	No of isolates, Incidence rate	No of isolates, Incidence rate
E. coli serogroup O:16	10/54 (18.5%)	16/72 (22.2%)	26/126 (20.6%)
E. coli Serogroup O:2	7/54 (12.9%)	13/72 (18.1%)	20/126 (15.9%)
E. coli Serogroup O:6	7/54 (12.9%)	9/72 (17.4%)	16/126 (12.7%)
Untypable *E. coli*	6/54 (11.1%)	8/72 (12.5%)	14/126 (11.1%)
Total *E. coli*/ cases	30/54 (55.6%)	46/72 (63.9%)	76/126 (60.3%)

* n = The number of examined animals.

Table 2: Hemagglutination pattern of *E. coli* serogroups on different erythrocytes

Source of erythrocytes	*E. coli* serogroup O:16 (n=26)*		*E. coli* serogroup O:2 (n=20)		*E. coli* serogroup O:6 (n =16)	
	Mannose resistant	Mannose sensitive	Mannose resistant	Mannose sensitive	Mannose resistant	Mannose sensitive
Horse	20/26 67.9%	4/26 15.4%	15/20 75%	5/20 25%	15/16 93.8%	7/16 43.6%
Bovine	12/26 46.2%	7/26 26.9%	12/20 60%	6/20 30%	7/16 43.8%	5/16 31.3%
Chicken	4/26 15.4%	1/26 3.8%	7/20 35%	5/20 25%	4/16 25%	1/16 6.3%
Human	3/26 11.5%	12/26 46.2%	3/20 15%	16/20 80%	2/16 12.5%	7/16 43.6%

*n = The number of *E. coli* i solates from each serogroup.

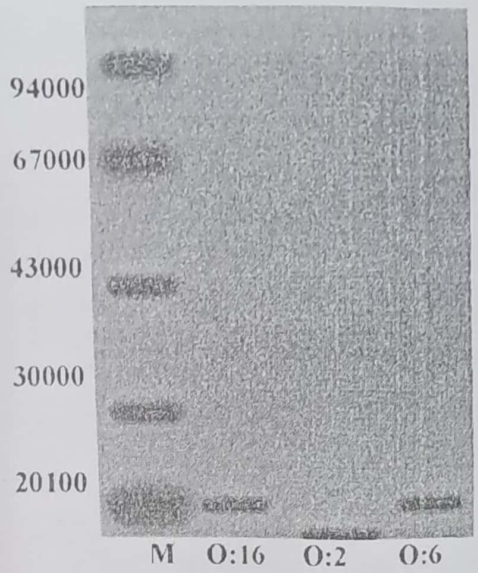

Figure 1: SDS-PAGE of pili preparation from the different *E. coli* serogroups.

Coomassie blue-stained SDS-PAGE profiles of purified pilus protein preparations from three *E. coli* serogroups, O:16, O:2, O6. Low molecular weight standard was used (Pharmacia Trypsin inhibitor 20.1kDa; Carbonic anhydrase 30kDa; Ovalbumin 43kDa; Albumin 67kDa and Phosphorylase b.

Table 3: The pattern of adhesion of different *E. coli* serogroups on UTEC and the effect of antipili antibodies on adhesion capacity.

E. coli serovar	Adhesion on UTEC (n = 20)*	Adhesion inhibition by homologous antibodies	Adhesion inhibition by heterologous antibodies	% of inhibition in correlation to homologous results
O:16	74.3 ± 8.5**	10.5 ± 3.7**	(O:2) 57.7 ± 8.9** (O:6) 28.3 ± 3.2	85.9%
O:2	68.5 ± 12.4	7.3 ± 2.9	(O:16) 54.2 ± 9.6 (O:6) 49.4 ± 11.7	89.3%
O:6	54.7 ± 5.7	6.4 ± 4.1	(O:16) 18.6 ± 5.4 (O:2) 47.1 ± 7.3	88.5%

* n = number of UTEC

**Average number of adherent bacteria/UTEC ± standard deviation.

Table 4: The effect of antipili antibodies in the protection of rabbits against challenge with 3 *E. coli* serogroups.

Antipili type	Type of challenge	Average No. of cfu/g kidney in immunized rats	Average No. of cfu/g kidney in non immunized rats
O:16 O:16 O:16	Homologous strain O:16 Heterologous strain O:2 Heterologous strain O:6	0 $1.2x10^3$ >10	$2.4x10^5$ infected with serogroup O:16
O:2 O:2 O:2	Homologous strain O:2 Heterologous strain O:16 Heterologous strain O:6	0 $2.3x10^3$ $1.7x10^4$	$1.8x10^5$ infected with serogroup O:2
O:6 O:6 O:6	Homologous strain O:6 Heterologous strain O:16 Heterologous strain O:2	0 >10 $1.9x10^3$	$2.7x10^5$ infected with serogroup O:6

In the in-vitro adherence test of *E. coli* isolates to UTEC, all *E. coli* isolates were highly adherent to UTEC especially the isolates of the serogroup O:16 (Table 3) In the adhesion inhibition test using the homologus antipili antisera, the number of *E. coli* adhered to UTEC was significantly ($P<0.01$) reduce, whereas the hetrologus antibodies could reduce the adhesion in case isolates of the serogroup O:16 and O:6 only, while heterologus antipili antisera could not inhibit the adhesion of the isolates from the serogroup O:2 (only the homologus antipili could inhibit the adhesion) (Table 3).

When white New Zealand rabbits were passively immunized with antipili hyperimmune serum that showed a high agglutination titer (1:640 in serogroup O:16 and O:6 while 1:1280 in serogroup O:6), they tolerated the infection when challenged with homologus *E. coli* isolates. In case of heterologus challenge there was a cross protection between serotvar O:16 and O:6 and the adhesion was also significantly reduced ($P<0.01$), whereas the group of rabbits passively immunized with hyperimmune serum against serogroup O:2 were not protected when challenged with isolates of serogroup O:16 or O:6 (Table 4).

DISCUSSION

Bacterial pathogens invading the urinary tract may induce symptomatic infection including the kidney as pyelonephritis or the urinary bladder as cystitis. Bacteriurea may also be accompanied without prominent symptoms. Many factors affect the severity of the urinary tract infection as the virulence characteristics of the bacterial strains involved in the infection and their ability to attach

and colonize the urinary tract. (Salyres and Whitt 1994; Linhares et al., 1999; Hamrick et al., 2000 and Sauvonnet et al., 2000) .

In this study the bacteriological examination of 126 mid-stream urine samples from the clinical cases revealed the isolation of *E. coli* from 76 cases which constituted 60.3% of the cases, other bacteria were also isolated but in lower pattern as *Streptococcus* spp. 14 cases (11.1%); *Klebsiella* spp. 12 cases (9.5%); *Proteus* spp. 11 cases (8.7%); *Staphylosoccus* spp. 7 cases (5.6%); *Corynebacterium* 3 cases (2.3%). Mares were more susceptible to urinary tract infection than stallions this could be attributed to the anatomical and physiological features of the female urinary tract which has a wider uretheral diameter and located in the vagina at close proximity from the anal opening which allows the bacterial pathogens to get easy access to the urinary tract. The *E. coli* was isolated from (63.9% mares) and from (55.6%) stallions. These findings supported the previous investigations of Hagberg et al., (1981), Orskov et al., (1982); Held et al., (1986); Salyres and Whitt (1994). These *E. coli* isolates belonged to three serogroups O:16 (26 isolates, 20.6%), O:2 (20 isolates, 15.9%), and O:6 (16 isolates, 12.7%) in addition to another 14 isolates that were untypable (11.1%) (Table1).

The SDS-PAGE analysis of the pili preparations from each serogroup revealed one distinct protein band 20.1kDa specific for the serogroup O:16 and O:6, whereas a 16.6kDa band was for the serogroup O:2 (Figure1); the similarity of the protein band of both O:16 and O:6 could be suggestive that both serogroups could carry the same pilus type which proved to be true due to their similarity in the hemagglutination pattern and their adhesion to UTEC. The obtained results agreed with Klemin and Oraskov (1982) and Suwanichkul et al., (1987) who identified similar pilus protein bands from uropathogenic strains of *E. coli* with small quantities of non pilus protein contaminants, that did not interfere with pili chemical characteristics.

The hemagglutination activity of *E. coli* isolates to the different erythrocyte types was similar and their corresponding purified pili preparations. Most of the *E. coli* isolates showed mannose resistent (MR) hemagglutination to horse RBS regardless of their serogroup, which was explained by many authors as it was due to the presence of pili typeII (Ofek and Beachey 1978; Orskov et al., 1982 and Svanborg-Eden et al., 1982) Hemagglutination to bovine and chicken RBCs was more to the expression of the MR hemagglutination than to the mannose sensitive (MS), while the agglutination of the human RBCs by all the three serogroups was more of the (MS) type which was correlated to the presence of pili typeI (Table 2).

These hemagglutination characteristics were explained as a complex interaction between the ligand (pili) on the *E. coli* cell and the carbohydrate

residues which is basically mannose on the RBCs. Many bacteria contain both MR and MS pili on their structure which explain the variation in hemagglutination results, moreover in many cases the MS pili after subculturing on agar media it changes to MR which is a reason for discrepancies in results. Many other factors were found to alter the chemical features of the pili.(Orskove et al., 1980; Hagberg et al., 1981; Korhonen et al., 1982 and Hamrick et al., 2000).

In the in-vitro adherence test of *E. coli* isolates to UTEC, all *E. coli* isolates were highly adherent, especially the isolates of the serogroup O:16 (74.3 ± 8.5 bacteria/cell) the serogroup O:2 and O:6 were slightly less adherent. (Table 3). In the adhesion inhibition test using the homologus antipili antisera, the number of *E. coli* adhered to UTEC was significantly ($P<0.01$) reduced by 85.9%, 89.3% and 88.5% in the three serogroup isolates O:16, O:2 and O:6 respectively. The heterologus antibodies of the serogroups O:16 and O:6 could convey cross protection against heterologous challenges with isolates from the serotype O:16 and O:6 but not to isolates from the serotype O:2, only the homologus antipili of the serogroup O:2 could inhibit their adhesion (Table 3). This confirms the previous hypothesis that the pili from the serogroup O:16 and serogroup O:6 could be identical which was proved by the SDS-PAGE and their hemagglutination pattern. Other workers proved similar results that antipili antisera raised in rabbits could inhibit the adhesion of the homologous strains of *E. coli* on the UTEC and either partly or not at all inhibit the adhesion of the heterologous strains (Silverblatt et al., 1982 and Greene et al., 2001) which elucidated that, antisera to whole pili aggregated and distorted pili from homologous strains, but pili from heterologus strains were unaffected, whereas antisera to cleaved pilin resulted in partial aggregation to homologous and heterologous pili, suggestive to heterospecific antibodies when more pilus epitopes were exposed due to the cleavage.

When white New Zealand rabbits were passively immunized with antipili hyperimmune serum with a high agglutination titer (1:640 in serogroup O:16 and O:6 while 1:1280 in serogroup O:6), they tolerated the infection when challenged with homologus isolates. In case of heterologous challenge there was a cross protection between serotvar O:16 and O:6 and the adhesion was also significantly reduced ($P<0.01$),whereas the group of rabbits passively immunized with hyperimmune serum against serogroup O:2 were not protected when challenged with isolates of serogroup O:16 or O:6(Table 4). Pilus protein might contain various antigenic determinants that elicit high titer antibodies that could deter completely or partially the adhesion on the host cells in a dose response relationship depending on the degree of relatedness between the isolates (Stenquist et al., 1982; Chen et al., 1999; Langermann et al., 2000; Yabin et al., 2000 and Greene et al., 2001).

these data which confirmed the potential humoral immune response elicited by the pilus proteins and its crucial effect on inhibition of adhesion on epithelial cells, led to production of many commercial vaccine preparations (multivalent vaccines) containing various units from several antigenically distinct bacterial strains in an attempt to increase the spectrum of the induced immune response (Chen et al., 1999; Langermann et al., 2000 and Greene et al., 2001).

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