Phenotypic characterization and genomic DNA polymorphisms of *Escherichia coli* strains isolated as the sole micro-organism from vaginal infections

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Vaginal infections such as vulvovaginal candidiasis, trichomoniasis and bacterial vaginosis are common worldwide. Accurate diagnosis and prescription of appropriate treatments are important since these infections are linked to adverse outcomes for women during pregnancy and for newborns. Several aetiological agents are responsible for these infectious diseases; however, the presence of *Escherichia coli* in these infections is controversial. Thus, it is important to identify some phenotypic and genotypic properties of *E. coli* strains isolated from vaginal infections. Forty-six *E. coli* strains isolated from vaginal fluid as the sole micro-organism, and 20 other *E. coli* strains isolated from other samples (urinary tract infections, otitis and septicaemia) were analysed by several phenotypic tests. In addition, genotypic features were studied by RAPD-PCR techniques. Biochemical tests showed that the *E. coli* strains isolated from vaginal fluid could be grouped into a single cluster which is subdivided into two phenogroups. Analysis of the dendrogram based on fragment length polymorphisms of genomic DNA indicated that *E. coli* isolates from vaginal infections form a single cluster with two subdivisions. Further studies are needed to analyse the molecular structure and virulence characteristics of these *E. coli* strains in order to determine their potential role in vaginal infections.

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INTRODUCTION

The vagina is a complex habitat for micro-organisms, and vaginal infections are considered to be among the main causes of morbidity around the world. Studies of the microbial flora of the human vagina reveal that micro-organisms normally present in this habitat play an important role in preventing colonization by exogenous bacterial species (Donders *et al.*, 2000; van De Wijgert *et al.*, 2000). The vaginal microflora is usually constituted by 5–15 different bacterial species (Obata-Yasuoka *et al.*, 2002), normally characterized by a high number of *Lactobacillus* species. *Lactobacillus crispatus* and *Lactobacillus jensenii* are of great importance in maintaining the vaginal pH at approximately 4.7 (or less), which causes growth inhibition of other micro-organisms (Marrazzo, 2003).

Three major types of infections that affect the vaginal habitat are vulvovaginal candidiasis, trichomoniasis and bacterial vaginosis. The first two have been extensively characterized in the literature (Marrazzo, 2003). Bacterial vaginosis is the most common cause of vaginal discharge (Klebanoff *et al.*, 2004) and is described as a disequilibrium in the vaginal microflora in which the normally predom-

inant *Lactobacillus* species are overgrown by anaerobic and facultative species (Ness *et al.*, 2005). This condition is not associated with vaginal inflammation as occurs in vaginitis. Vaginal infections, but principally bacterial vaginosis, have been associated with many complications, including second trimester miscarriage, pelvic inflammatory disease, preterm birth, preterm premature rupture of the membranes, chorioamnionitis, post-partum endometritis, post-operative infection after gynaecological surgery and easier acquisition of HIV (Beigi *et al.*, 2004; Ness *et al.*, 2004). The aetiological agents of vaginal infection are *Candida albicans* in vulvovaginal candidiasis, and *Trichomonas vaginalis* in trichomoniasis. The predominant micro-organisms that cause bacterial vaginosis are *Gardnerella vaginalis*, *Mycoplasma hominis*, *Ureaplasma urealyticum* and others such as *Prevotella* spp., *Mobiluncus* spp. and *Bacteroides* spp. (Klebanoff *et al.*, 2004).

Escherichia coli is a commensal inhabitant of the intestinal tract of healthy humans and different animals (Clermont *et al.*, 2000). It is accepted that pathogenic *E. coli* strains are derived from commensal strains by acquisition of chromosomal or extrachromosomal virulence operons, and although extraintestinal pathogenic *E. coli* strains are mostly opportunistic, they can cause a diverse spectrum of serious diseases (Escobar-Páramo *et al.*, 2004; Sasakawa & Hacker, 2006). The presence of vaginal *E. coli* is

Abbreviations: RAPD, random amplified polymorphic DNA; UTI, urinary tract infection(s).

controversial with regard to its potential pathogenic role. Some studies report its presence as an opportunistic micro-organism (Watt *et al.*, 2003). Others concluded that this species has not been detected on healthy vaginal epithelium (Hyman *et al.*, 2005), and also it has been isolated from aerobic vaginitis. This infection is a condition of abnormal vaginal flora that differs from bacterial vaginosis. Genital complaints are those of a real vaginitis. Group B streptococci, *E. coli*, *Staphylococcus aureus* and *T. vaginalis* are frequently cultured (Donders *et al.*, 2002). Considering the serious obstetric and neonatal complications, and that about 30% of women with vaginal complaints remain undiagnosed despite extensive testing (Anderson *et al.*, 2004), it is of great importance to determine whether *E. coli* strains isolated as the sole micro-organism from vaginal infections have particular characteristics. Thus, the aims of this study were to identify *E. coli* strains isolated as the sole micro-organism from women of reproductive age and presenting vaginal infections, to analyse their phenotypic characteristics by means of biochemical tests, and to determine potential genotypic association among them. *E. coli* strains isolated in association with other known aetiological agents of vaginal infections were not considered in the present study.

METHODS

Study population. The study population was composed of 425 women of fertile age (18 to 41 years) who attended Talca City Hospital with clinical diagnosis of vaginal infection; they were non-pregnant, non-smokers, had no history of sexually transmitted infections, and had had one sexual partner during the previous 6 months. Also, they had had no antimicrobial treatment within the previous 6 months as revealed by a written questionnaire. In addition, they showed absence of menstruation and had no diarrhoea or urinary tract infections (UTI). Diagnosis of vaginosis was based on detection of three or more Amsel criteria (Amsel *et al.*, 1983) and the microscopic record of bacterial morphology to detect bacterial vaginosis (Nugent *et al.*, 1991). The study protocol was accepted by the Universidad de Talca Bioethical Committee and included an informed consent from the patients, before collecting clinical samples. As control samples, vaginal epithelium from 100 healthy women was analysed by microbiological procedures.

Sample collection and microbiology study. Vaginal swabs (three swabs per patient) were taken from the lateral and posterior vaginal fornix. One was immediately streaked on *Brucella* agar (Difco) supplemented with sheep blood (5%), vitamin K ($1 \mu\text{g ml}^{-1}$) (Merck), haemin ($5 \mu\text{g ml}^{-1}$) (Merck) and kanamycin ($50 \mu\text{g ml}^{-1}$) (Sigma) with the purpose of obtaining growth of *Bacteroides fragilis*, *Fusobacterium nucleatum*, *Porphyromonas gingivalis* and *Prevotella intermedia*. Plates were incubated at 37°C for 12 days in an anaerobic system (bioMérieux). The same swab was shaken into tubes with Schaedler broth (BD) and incubated anaerobically at 37°C for 7 days to demonstrate growth of *Mobiluncus* spp. A second swab was streaked onto MacConkey agar (Difco) and Trypticase Soy agar (Difco) supplemented with sheep blood (5%) and incubated in aerobic conditions at 37°C for 24 h. The same swab was streaked onto Thayer Martin agar (Difco) and incubated in the presence of CO_2 (5%) at 37°C for 48 h. Finally, the swabs were used to inoculate a Sabouraud agar plate (Merck) and on *Trichomonas* medium (Oxoid), for *Trichomonas vaginalis* growth. The third swab was used

with the *Mycoplasma* IST-2 kit (bioMérieux) to detect the growth of *Mycoplasma hominis* and *Ureaplasma urealyticum*. Finally the same swab was used to detect *Chlamydia trachomatis* with the TestPack *Chlamydia* kit (Abbot). Identification of *Enterobacteriaceae* family members was done with the API 20-E test (bioMérieux) and anaerobic bacteria were identified by phenotypic methods (Jousimes-Somer & Sumanen, 1999). *Candida albicans* was identified by the ID 32C kit (bioMérieux).

In order to compare phenotypic and genotypic characteristics of vaginal *E. coli* strains with other *E. coli* isolates from other origins, 15 *E. coli* isolates from non-pregnant women with UTI, and four *E. coli* isolates from blood samples of patients with septicæmia and one from a patient with otitis were used. These isolates, obtained with informed consent from patients (as described above), were identified by the same procedures as used with isolates from vaginal infections.

Determination of DNA polymorphism in *E. coli* strains isolated from vaginal infections by RAPD-PCR. Phylogenetic relationships among the *E. coli* strains isolated from vaginal infections and those used to compare phenotypic and genotypic characteristics were studied by means of random amplified polymorphic DNA (RAPD). This method has become a highly valuable molecular tool for intra-species characterization of *E. coli* (Cave *et al.*, 1994; Wang *et al.*, 1993). Genomic DNA was extracted from a single colony using the Aqua Pure Genomic DNA Isolation kit (Bio-Rad) and the DNA concentration was spectrophotometrically determined (A_{260}). PCRs were conducted in $25 \mu\text{l}$ reaction mixture containing 25 ng DNA template, 1 U *Taq* DNA polymerase (Invitrogen) in $1\times$ buffer, 300 μM of each dNTP and 0.8 nM of each primer. The primers used were 2H (5'-AAGCTTCGACTGT-3') and 3H (5'-AAGCTTGA-TTGCCC-3), which were randomly selected among four arbitrary primers. Amplification was done in a DNA Engine (Bio-Rad) thermal cycler programmed to one cycle of 5 min at 94°C , 10 cycles of 1 min at 94°C , 1 min at 36°C , and 2 min at 72°C followed by 20 cycles of 1 min at 94°C , 1 min at 50°C and 2 min at 72°C to allow the completion of DNA extension (Aslam *et al.*, 2004). A negative control, consisting of the same mixture but using water instead of DNA, was included in each run. This procedure was repeated twice per strain. Only fragments present in both amplifications were used to define the RAPD patterns. The amplification products were analysed by electrophoresis in 1% agarose gel, stained with ethidium bromide ($0.5 \mu\text{g ml}^{-1}$) and photographed under UV light. A 1 kb DNA ladder (New England Biolabs) was included as molecular size marker. The RAPD patterns obtained from *E. coli* strains isolated from vaginal infections were compared to those obtained from *E. coli* strains isolated from UTI, septicæmia and otitis. The numerical analysis was carried out using Taxotron software (Taxolab) (Chatellier *et al.*, 1997). For each strain, the RAPD type was defined as the combination of the band pattern obtained with both primers. The relationships among the RAPD types of the strains were calculated by the unweighted pair-group method averages method and represented as a dendrogram.

Statistical analysis. Significance of differences between variables was tested by means of χ^2 test and Fisher's exact test.

RESULTS

Bacteriological analysis of vaginal fluids

Table 1 shows that *Gardnerella vaginalis* was the most frequent micro-organism isolated (33.2%) followed by *E. coli* (23.0%) in the 425 women with clinical diagnosis of vaginal infection. Other micro-organisms frequently

Table 1. Micro-organisms isolated from women with vaginal infections and from the vagina of healthy women

Micro-organism	Vaginal infections			Healthy women <i>n</i> (%)
	Total <i>n</i> (%)	Vaginitis <i>n</i> (%)	Vaginosis <i>n</i> (%)	
<i>G. vaginalis</i>	141 (33.2)	12 (2.8)	129 (30.3)	26 (26)
<i>E. coli</i>	96 (23.0)	27 (6.3)	69 (16.2)	6 (6)
<i>C. albicans</i>	66 (15.5)	66 (15.5)	0	0
<i>T. vaginalis</i>	39 (9.1)	39 (9.1)	0	0
<i>Mobiluncus</i> spp.	12 (2.8)	0	12 (2.8)	6 (6)
<i>Ent. faecalis</i>	10 (2.4)	0	10 (2.4)	2 (2)
GNAB*	9 (2.2)	0	9 (2.2)	4 (4)
<i>M. hominis</i>	5 (1.2)	0	5 (1.2)	0
<i>C. trachomatis</i>	2 (0.5)	2 (0.5)	0	0
<i>S. agalactiae</i>	3 (0.7)	0	3 (0.7)	8 (8)
NGM†	42 (9.9)	14 (3.3)	28 (6.6)	56 (56)
Total	425 (100)	160 (37.5)	265 (62.4)	100 (100)

*GNAB, Gram-negative strictly anaerobic bacilli (*B. fragilis*, *P. gingivalis*, *P. intermedia*, *F. nucleatum*).

†NGM, No growth of micro-organisms, but existing growth of *Lactobacillus* spp. and coagulase-negative *Staphylococcus*.

isolated were *Candida albicans* and *Trichomonas vaginalis*, 15.5% and 9.1%, respectively. Gram-negative strictly anaerobic bacilli were isolated in only 2.2% of the cases. No growth of *Neisseria gonorrhoeae* or *Ureaplasma urealyticum* was observed. Moreover, in 42 of the samples studied no bacterial growth was detected. Also, 26 vaginal samples from healthy women yielded *G. vaginalis* strains and a low growth of *E. coli*, *Mobiluncus* spp., *Enterococcus faecalis*, *Streptococcus agalactiae* and Gram-negative strictly anaerobic bacilli (*Bacteroides fragilis*, *Porphyromonas gingivalis*, *Prevotella intermedia* and *Fusobacterium nucleatum*). Table 2 shows micro-organisms recovered from women with vaginal infection. *E. coli* strains appeared associated with *G. vaginalis*, *C. albicans* and *T. vaginalis* in 29, 12 and 8 samples of vaginal fluid respectively. Individually, *G. vaginalis* was the most frequently isolated micro-organism, from 97 samples, and *E. coli* was isolated as a single micro-organism in 46 samples.

Phenotypic properties of the *E. coli* strains

A comparison of the biochemical test results obtained for *E. coli* isolates from vaginal specimens and from other sources is shown in Table 3. Tests for ornithine decarboxylase ($P \leq 0.001$), L-rhamnose ($P \leq 0.022$) and sucrose fermentation ($P \leq 0.0001$) showed significant differences between the two groups of *E. coli* strains.

RAPD analysis of *E. coli* strains isolated from vaginal fluid as the sole micro-organism

RAPD analysis of genomic DNA of the 46 *E. coli* strains isolated as the sole micro-organism from vaginal samples resulted in 63 profiles when using primers 2H and 3H. A profile of ten and seven polymorphic fragments was observed with the 3H and 2H primer set, respectively.

The molecular masses of the fragments obtained with the 3H primer ranged between 500 and 4500 bp and the fragments produced by the 2H primer ranged between 500 and 6000 bp (data not shown). A total of 46 RAPD profiles were observed among vaginal isolates and 17 among isolates from other sources. The dendrogram presented in Fig. 1 shows three main clusters, labelled A, B and C. Cluster A included a single isolate from otitis. Cluster B encompassed the 46 *E. coli* strains of vaginal origin plus four strains from septicaemia, and cluster C included the

Table 2. Number of samples from women with vaginal infection that yielded growth of associated and individual micro-organisms.

Micro-organism(s)	No. of samples
<i>G. vaginalis</i> + <i>E. coli</i>	29
<i>C. albicans</i> + <i>E. coli</i>	12
<i>T. vaginalis</i> + <i>E. coli</i>	8
<i>G. vaginalis</i> + <i>Mobiluncus</i> spp.	6
<i>G. vaginalis</i> + <i>C. albicans</i>	5
<i>G. vaginalis</i> + GNAB*	4
<i>G. vaginalis</i>	97
<i>C. albicans</i>	49
<i>E. coli</i>	46
<i>T. vaginalis</i>	31
<i>E. faecalis</i>	10
<i>Mobiluncus</i> spp.	6
<i>M. hominis</i>	5
GNAB*	5
<i>S. agalactiae</i>	3
<i>C. trachomatis</i>	2

*GNAB, Gram-negative strictly anaerobic bacilli (*B. fragilis*, *P. gingivalis*, *P. intermedia*, *F. nucleatum*).

Table 3. Phenotypic characteristics of *E. coli* strains isolated from women with vaginal infections as the sole micro-organism in comparison with *E. coli* strains from other sources

Tests positive for all strains: ONPG, indole production, D-glucose acid, D-mannitol fermentation, D-sorbitol fermentation, D-arabitol fermentation, nitrate to nitrite and lysine decarboxylase. Tests negative for all strains: citrate (Simmons), hydrogen sulfide, urea hydrolysis, tryptophan deaminase, Voges–Proskauer (acetoin), gelatin hydrolysis and oxidase.

Biochemical test	<i>E. coli</i> strains			
	Vaginal source (<i>n</i> =46)		Other source (<i>n</i> =20)	
	No.	(%)	No.	(%)
Melibiose fermentation	45	(97)	20	(100)
L-Rhamnose fermentation	44	(95)	15	(75)
Ornithine decarboxylase	29	(63)	20	(100)
Sucrose fermentation	24	(52)	20	(100)
Arginine dihydrolase	16	(34)	3	(15)
Amygdalin fermentation	2	(4)	0	(0)
<i>myo</i> -Inositol fermentation	1	(2)	0	(0)

isolates from UTI. The B cluster was subdivided into two genogroups (B1, 23 strains, and B2, 20 strains), both with *E. coli* strains of vaginal origin. Genogroups B1 and B2 represented 93.5 % of all *E. coli* strains obtained from a vaginal source.

DISCUSSION

Vaginal infections are still a major cause of morbidity, leading to millions of dollars spent worldwide annually in physician services and medications. Vaginal infections are not reportable diseases, and therefore accurate estimates of incidence are unavailable (Schwebke, 2000). Infections usually respond to appropriate treatments; however misdiagnosis and pharmacological failure may occur. Therefore, it is important to diagnose the problem accurately and prescribe the appropriate treatment (Owen & Clenney, 2004). The high prevalence of vaginal infections, along with controversies with regard to aetiological agents, may be related to unidentified bacteria as agents of such infections. The present study establishes differences among *E. coli* strains isolated from different human habitats, including the vagina, and identifies an association between strains isolated as the sole micro-organism from women with vaginal infections with regard to both phenotypic and genotypic properties. This finding supports a potential role of particular subgroups of *E. coli* in the pathogenesis of vaginal infections.

The aetiological role of *E. coli* in vaginal infections has been controversial. In general, their isolation from vaginal fluids is considered as normal, and women are not treated. Our

study of vaginal specimens from 425 women with clinical diagnosis of vaginal infection showed 62.4 % of bacterial vaginosis cases in comparison to 37.5 % of vaginitis, in agreement with previous reports (Klebanoff *et al.*, 2004; Beigi *et al.*, 2004). Moreover, it is important to observe that *T. vaginalis* and *C. albicans* were restricted to vaginitis infection. *E. coli* was present in both bacterial vaginosis and vaginitis (23 % of total bacterial isolates), which made it interesting to determine if *E. coli* strains can be associated with other aetiological agents. In this study *E. coli* was isolated as the sole micro-organism in 46 cases. The absence of other recognized aetiological agents of vaginal infection made these bacterial strains an interesting research topic.

When the strains from vaginal, UTI, septicaemia and otitis sources were analysed for ornithine decarboxylase, sucrose fermentation and rhamnose fermentation, only those isolated as the sole micro-organism from vaginal infections showed significant particular activities. Thus, only 63 % of these *E. coli* strains showed positive activity for ornithine decarboxylase, and 95 % and 52 % showed positive activity on rhamnose and sucrose, respectively (versus 100 %, 75 % and 100 %, respectively, for the strains from other sources). It is important to note that phenotypic and chemotaxonomic characteristics are indirect markers of genomic changes (Coenye *et al.*, 2005).

Results obtained by means of RAPD showed different profiles of genomic DNA. These molecular profiles were highly reproducible, allowing the analysis of different genotypic profiles by means of a dendrogram. The results showed that the *E. coli* strains grouped in three clusters. The strains of vaginal origin were clustered together in a distinct cluster, subdivided into two genogroups, which constituted 93.5 % of the total vaginal isolates. This suggests that these strains, isolated as the sole micro-organism from vaginal infections, constitute a separate subpopulation within the species. Our study further demonstrated that *E. coli* strains isolated from UTI are closely related to the strains of vaginal origin. Other studies have suggested that *E. coli* vaginal residents present a common phylogeny to uropathogenic *E. coli* strains (Obata-Yasuoka *et al.*, 2002) and also that uropathogenic *E. coli* are better adapted than other *E. coli* to the urethra, periurethra and vagina (Foxman *et al.*, 2002). It is possible that in the referenced studies *E. coli* was not isolated as the sole micro-organism from vaginal infections and was not causally associated with the vaginal infection.

According to our results, it is plausible to postulate that the *E. coli* strains isolated as the sole micro-organism from the vagina may belong to a particular phylogenetic group. This could be based on the high genomic plasticity of *E. coli* and its capacity to acquire or eliminate genes. Since micro-organisms are defined by their environment, it would be unlikely that micro-organisms capable of causing human diseases evolve in such an environment where there are no humans or other animals (Wilson & Salyers, 2003). This is

Fig. 1. Genetic relationships among the *E. coli* strains studied and the respective biochemical tests used for vaginal *E. coli* (0, negative biochemical test; 1, positive biochemical test). ADH, arginine dihydrolase; LDC, lysine decarboxylase; ODC, ornithine decarboxylase; INO, *myo*-inositol fermentation; RHA, L-rhamnose fermentation; SAC, sucrose fermentation; MEL, melibiose fermentation.

likely to apply with respect to mutations that lead to increasing virulence, considering that the amplification of those mutations requires a selective pressure, presumably provided by the host environment, the vagina habitat in this case.

Many genital infections occur as a result of new sexual practices, involving significant microbiological aspects. This could explain the presence of new aetiological agents of vaginal infections or the adaptation of micro-organisms such as *E. coli*. Finally, the potential role of *E. coli* in vaginal infections deserves further molecular studies to learn more about the particular subset of this species that appears to be aetiologically involved.

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