

Genetic relationship between *Escherichia coli* strains isolated from dairy mastitis and from the stable fly *Stomoxys calcitrans*¹

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ABSTRACT- Castro B.G., Souza M.M.S., Regua-Mangia A.H. & Bittencourt A.J. 2016. **Genetic relationship between *Escherichia coli* strains isolated from dairy mastitis and from the stable fly *Stomoxys calcitrans*.** *Pesquisa Veterinária Brasileira* 36(6):479-484. Universidade Federal de Mato Grosso, Campus Universitário de Sinop, Av. Alexandre Ferronato 1200B, Setor Industrial, Sinop, MT 78557-270, Brazil. E-mail: castrobg@ufmt.br

The stable fly *Stomoxys calcitrans* (Linnaeus, 1758) has been described as a potential spreader of infectious agents to cattle herds. Among the agents transmitted by this fly, *Escherichia coli* has attracted attention due to its potential to cause gastrointestinal disorders as well as environmental mastitis in dairy cows. Therefore, the aim of this study was to isolate and to assess the genetic diversity and the clonal relatedness among *E. coli* isolates from the milk of dairy mastitis and from stable flies anatomical sites by the Random Amplification of Polymorphic DNA (RAPD-PCR) technique. The molecular typing revealed a high degree of genetic polymorphism suggesting that these microorganisms have a non-clonal origin. Identical electrophoretic profiles were observed between *E. coli* isolates from different flies, different mammary quarters of the same cow and from cows on a single farm. These results reveal the circulation of the same bacterial lineages and suggest the role of the stable fly in bacterial dispersion. Considering the high pathogenic potential of this bacterial species, our findings alert to a more effective health surveillance.

INDEX TERMS: Random amplification of polymorphic DNA, RAPD, milk, stable fly, clonal species.

RESUMO.- [Caracterização genotípica de *Escherichia coli* isolados de leite com mastite e da mosca dos estábulos *Stomoxys calcitrans*.] A mosca dos estábulos *Stomoxys calcitrans* é descrita como um importante dispersor de agentes infecciosos aos bovinos. Dentre os agentes veiculados por esta mosca a bactéria *Escherichia coli* ganha relevância devido ao seu potencial em desenvolver alterações gastroentéricas, bem como mastite bovina ambiental. Desta forma, objetiva-se com este estudo isolar e acessar a diversidade genética e relação de clonalidade entre isolados de *E. coli* provenientes de casos de mastite e de moscas dos estábulos utilizando a técnica da Amplificação Randô-

mica do DNA Polimórfico (RAPD). A tipagem molecular revelou elevado polimorfismo genético sugerindo que esses microrganismos têm origem não clonal. Perfis eletroforéticos idênticos entre si foram observados entre amostras isoladas de diferentes moscas, quartos mamários de uma mesma vaca, bem como de diferentes vacas dentro de uma mesma propriedade. Esses resultados revelam a circulação de uma mesma linhagem bacteriana e sugerem o papel da *Stomoxys calcitrans* na dispersão bacteriana. Considerando o elevado potencial patogênico dessa espécie bacteriana, nossos achados alertam para uma vigilância sanitária mais efetiva.

TERMOS DE INDEXAÇÃO: Amplificação randômica do DNA polimórfico, RAPD, leite, mosca dos estábulos, clones.

INTRODUCTION

Despite the reduction in the number of cases of bovine mastitis of contagious origin in the world, several authors have reported that cases of environmental origin have been increasing, mainly caused by *Escherichia coli* (Fairbrother et al. 2015). The proportion of *E. coli* isolated as an etiolo-

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gical agent from the milk of cows suffering from mastitis varies according to the place of study. According to Nevala et al. (2004), in Finland fewer than 20% of mastitis cases are caused by *E. coli*, while Shpigel et al. (1998) and Shpigel et al. (2008) reported 60% in Israel, as well as the *E. coli* is the leading cause of acute mastitis in dairy animals in that country. In Brazil the cases of mastitis caused by *E. coli* have a prevalence ranging from 4.0% to 15%, possibly due to the stock-raising system used in the country, where the animals generally are confined to stables only at the time of milking, thus reducing the possibility of contaminating the mammary quarters by feces (Ferreira et al. 2007).

Hyper-acute and acute udder infections by *E. coli* occur in the first weeks of clinical and sub-clinical form. They are difficult to cure therapeutically, and cases of systemic involvement can lead to death by toxemia (Hertl et al. 2010). The main source of contamination is by direct contact of the recently milked mammary quarters with materials contaminated by the agent, such as feces, unhygienic equipment, dirty hands of stable workers and possibly some insects, as muscid flies (Pyörälä 2002, Ryman et al. 2013).

Some muscid flies have been studied for their potential to transmit agents causing mastitis. Among these, the flies *Hydrotea irritans*, *Haematobia irritans* and *Musca domestica* are most often studied in Europe and USA and have been implicated on carriage of bacterial mastitis strains in experimental cases (Chirico et al. 1997, Braverman et al. 1999, Anderson et al. 2012).

The stable fly has also been studied among the Muscidae family causing injury to Brazilian cattle herds. Some authors have found similarity in the isolation of agents from cases of bovine mastitis and in *Stomoxys calcitrans* (Castro et al. 2001, Moraes et al. 2004). However, these authors did not confirm the real potential of stable flies to transmit agents of bovine mastitis; they only reported the possibility of this occurrence.

This study was designed to isolate and to assess the genetic diversity among *E. coli* isolates from mastitic milk of dairy cattle and stable fly from dairy farms in Rio de Janeiro state.

MATERIALS AND METHODS

For this study 10 dairy farms in the municipalities of Barra Mansa and Resende located at Rio de Janeiro state, Brazil, were visited. These two municipalities were selected because these locations are the main dairy region of the state of Rio de Janeiro (IBGE 2009). Also, previous studies detected a high prevalence of the stable fly and in these municipalities it was identified common bacterial species from the microbiota of macerated flies and mastitic milk, but the genetic relationship was not evaluated (Castro et al. 2001, Moraes et al. 2004).

At the visited farms, the cows ready for milking were submitted to the California Mastitis Test (CMT). After detected the positive quarters, milk samples were taken from these after washing the teats with neutral soap and water and then drying and disinfecting them with iodized alcohol. The milk samples were placed in sterile tubes and taken under refrigeration to the laboratory for bacterial isolation.

Twenty flies were also collected at each farm, using an insect sweep net, giving preference to those that were feeding or flying

at most one half meter from the animals (Bramley et al. 1985, Puri-Giri et al. 2015). Each fly was placed in a sterile test tube and taken under refrigeration to the laboratory.

All the dissection procedures of the flies were performed under a fume hood near a Bunsen burner. The flies were identified according to Bowman (2014), and only the *S. calcitrans* specimens were killed by freezing at -10°C for five minutes. Then, each frozen fly was placed in a test tube containing enriched brain-heart infusion (BHI) Broth, agitated in this tube and transferred to another test tube containing 70% alcohol for 10 minutes for sterilization of the outer surface, as described by Hillerton & Bramley (1985) and Castro et al. (2013).

Then each fly was placed on its back in a sterile Petri dish and a stereoscopic microscope was used to aid removal of the mouth apparatus and abdominal digestive tract, as described by Castro et al. (2007). Each segment was then macerated in BHI Broth and incubated in a bacteriological chamber at 37 °C for at least 24 hours.

After the incubation period, the samples were transferred to Petri dishes containing the MacConkey agar and Eosin Methyl Blue (EMB) agar. The colonies were then observed regarding morphological and physiological characteristics. After differentiation of the typical *E. coli* colonies, a preliminary identification assays was carried out: Gram staining and the hydrolysis test to 3% KOH to confirm the Gram catalysis test. Putative *Escherichia coli* colonies were confirmed biochemically and the following identification tests were conducted: behavior in triple sugar iron agar; behavior in SIM agar; production of acids by glycolysis; sugar fermentation; Voges Proskauer (VP) test; methyl red (MR) test; nitrate reduction; gelatinase production; citrate and malonate degradation; and other differential tests according to the microorganism involved (Koneman et al. 2008).

E. coli was stored frozen for further genetic analysis. Prior to use, each isolate was streaked onto Trypticase Soy Agar (TSA; BBL) and grown overnight at 37°C for analysis of purity and viability. The genetic diversity was investigated by RAPD-PCR according to the methodology described by Pacheco et al. (1997) and Vogel et al. (2000).

For the bacterial typing, a colony of *E. coli* was incubated in Trypticase Soy Broth (TSB - BBL Microbiology Systems, Cockeysville, MD) at 37°C. After 18-24 hours, an aliquot of the bacterial culture was diluted 1:10 in water immediately before use to determine the optical density at 600 nm. For an optical density value of 0.4, a 200-µl aliquot of the stationary-phase culture was centrifuged (12,000 *g* for 2 min) and suspended in 900 µl of sterile distilled water. The suspension was then boiled for 10 min, briefly centrifuged, and the bacterial lysate was used as the template source in amplification assays. RAPD reactions were carried out with six 10-mer primers: 1247 (5'-AAGAGCCGT-3'), 1253 (5'-GTTTCCGCCC-3'), 1281 (5'-AACGCGCAAC-3'), 1254 (5'-CCGCAGCCAA-3'), M13 (5'-GAGGGTGGCGTTCT-3'), and A04 (5'-AATCGGGCTG-3'). A volume of 3 µL of the DNA template together with other reagents: 20 mM of Tris-HCl (pH 8.4) (Fermentas, Burlington, Canada), 50 mM of HCl, 4 mM of MgCl₂ (Fermentas, Burlington, Canada), 250 µM (each) of dNTP (ABgene, Epsom, UK), 30 pmol/µl of primer and 1U of Taq polymerase (Fermentas, Burlington, Canada) were used in the amplification reactions,

The reaction was programmed for initial denaturation at 94°C for 1 minute; followed by four cycles at 94°C for 5 minutes, 37°C for 5 minutes and 72°C for 5 minutes; 30 cycles at 94°C for 1 minute, 37°C for 1 minute and 72°C for 2 minutes; and final extension at 72°C for 10 minutes. Reaction products were loaded on 1.5% agarose gel and stained with ethidium bromide (0.5 µg/mL) after electrophoresis for 3 hours at 100V.

A 1Kb DNA ladder (Invitrogen Brasil,) was used as a size marker. RAPD-PCR profiles were defined according to the presence or absence, and intensity of polymorphic bands. Genetic patterns were compared by visual and automated analysis by using the UVIPro software (UVIbandmap version 11.9, UVITEC, Cambridge, UK). Cluster analysis was done by using the UPGMA method of the UVIPro software applying a maximum tolerance of 1.2% and the percentages of similarity were estimated by the Jaccard's coefficient. The reproducibility of the RAPD patterns was assessed by using the selected primers with different DNA samples isolated independently from the same strain amplified at different times.

RESULTS AND DISCUSSION

The investigation on the etiology of the microbiota in the milk and flies revealed that *Escherichia coli* was the sole bacterial species present in both biological specimens, found on farms 1, 2, 3, 4, 7, 9 and 10, for a total of 58 *E. coli* colonies. No other enterobacterial species was observed simultaneously in the stable flies and milk samples collected from a single farm. This can probably be explained by the fact that *E. coli* is the leading enterobacterium causing bovine mastitis, as reported in other studies (Lehtolainen 2004, Halasa et al. 2007). The observation of *E. coli* as the only species isolated both from milk and fly samples can be explained by the fact that this bacteria is most commonly implicated in episodes of environmental bovine mastitis, as commented by Blum et al. (2014).

In this study, six random primers were used (M13, 1247, 1254, 1253, 1281 and A04) and all of which had previously been utilized in *E. coli* population studies (Pacheco et al. 1997, Grif et al. 1998, Regua-Mangia et al. 2004). After initial amplification reactions, three of them (A04, 1254 and M13) were selected for RAPD typing since their profiles presented reproducibility, higher discriminatory power and distinct polymorphic bands.

RAPD typing revealed a genetically diverse bacterial population. Amplification reactions generated informative profiles composed of 4-21 polymorphic bands ranging from 320 bp to 3500 bp: primer A04 generated profiles ranging from 5 to 19 bands; primer 1254 profiles composed by 4 to 17 bands; and primer M13 profiles with 7 to 21 bands. The-

re was no clear correlation between C+G content and the ability of the primer to detect polymorphic. The reactions with primers A04 (60%), 1254 (70%) and M13 (66%) generated 49, 49 and 50 distinct profiles, respectively (Table 1 and 2).

Identical RAPD genetic profiles were observed between bacterial *E. coli* isolates from distinct cows on a single farm. The circulation of common genetic patterns provides evidence of the circulation of specific lineages between herds which possibly occurred due to the low level of milking hy-

Table 2. *Escherichia coli* samples identified and its electrophoretic profile from anatomical sites of stable flies collected from dairy farms of Barra Mansa and Resende municipalities, Rio de Janeiro State, Brazil

Sample	Farm	Fly number	Anatomical site	Electrophoretic profiles		
				1254	A04	M13
4	I	4	Body surface	2	2	2
5	I	18	Body surface	3	3	3
7	II	3	Body surface	5	5	5
8	II	9	Body surface	6	6	6
9	II	16	Mouth apparatus	7	7	7
11	III	10	Body surface	9	9	9
12	III	12	Body surface	10	10	10
13	V	2	Mouth apparatus	11	11	11
14	V	13	Body surface	12	12	12
15	V	13	Mouth apparatus	13	13	13
16	V	19	Abdominal digestive tract	13	13	13
24	IV	1	Body surface	18	18	18
25	IV	8	Mouth apparatus	19	19	19
26	IV	16	Body surface	20	20	20
28	VI	2	Abdominal digestive tract	21	21	21
29	VI	7	Abdominal digestive tract	22	22	22
30	VI	14	Body surface	23	23	23
33	VII	10	Body surface	25	25	25
34	VII	12	Mouth apparatus	26	26	26
35	VII	14	Body surface	27	27	27
36	VII	19	Mouth apparatus	28	28	28
37	VII	19	Body surface	28	28	29
38	VIII	14	Body surface	29	29	30
39	VIII	14	Mouth apparatus	29	30	31
40	VIII	14	Abdominal digestive tract	30	30	31
41	VIII	20	Body surface	30	30	31
44	IX	4	Body surface	32	32	33
45	IX	14	Mouth apparatus	33	33	34
46	IX	20	Mouth apparatus	34	34	35
49	X	2	Abdominal digestive tract	36	36	37
50	X	3	Mouth apparatus	37	37	38
51	X	5	Body surface	38	38	39
52	X	6	Mouth apparatus	39	39	40
53	X	7	Mouth apparatus	40	40	41
54	X	7	Abdominal digestive tract	41	41	42
55	X	9	Mouth apparatus	42	42	43
56	X	10	Body surface	43	43	44
57	X	11	Body surface	44	44	45
58	X	13	Body surface	45	45	46
59	X	15	Body surface	46	46	47
60	X	17	Body surface	47	47	48
61	X	19	Abdominal digestive tract	48	48	49
62	X	20	Body surface	49	49	50

Table 1. *Escherichia coli* samples identified and its electrophoretic profile from mastitic udders from dairy farms of Barra Mansa and Resende municipalities, Rio de Janeiro State, Brazil

Sample	Farm	Cow number	Udder teat	Electrophoretic profiles		
				1254	A04	M13
2	I	3	Rear left	1	1	1
3	I	5	Front right	1	1	1
6	II	12	Rear left	4	4	4
10	III	16	Rear right	8	8	8
19	IV	24	Front right	15	15	15
20	IV	24	Rear right	16	16	16
21	IV	26	Rear left	16	16	16
22	IV	27	Front left	16	16	16
23	IV	28	Rear right	17	17	17
31	VII	37	Front right	24	24	24
32	VII	37	Rear left	24	24	24
42	IX	43	Front left	31	31	32
43	IX	43	Front right	31	31	32
48	1X	49	Front left	35	35	36

giene at the farms visited as verified by Kempf et al. (2016). Careless milking practices were often observed, without routine cleaning of the teats and hand washing by the farm workers, practices that favor the spread of bacteria as reported by De Vlieghe et al. (2012). It was not possible to observe the use of prophylactic strategies as food supply to the cows after milking, to prevent them from lying down before closure of the streak canal, which, in turn allows the entrance of agents of mastitis in the mammary quarters. According to Oliver et al. (2001) and Lopes et al. (2012), prophylactic strategies decrease the prevalence of environmental and contagious mastitis.

Identical genetic patterns were shared by *E. coli* isolates from different parts of the same fly and from different flies, as observed with colonies from the body surface. This was possibly due to the presence of the bacterium in the environment used by the flies for development during their non-parasitic phases, as reported by Ahmed et al. (2007).

It was isolated *E. coli* strains from milk that exhibited identical electrophoretic profile from farms that shared certain characteristics, such as manual milking and precarious hygiene, with milking done in most cases in direct floor stalls. These results thus reinforce the reported by Costa Sobrinho et al. (2012) that the poor hygiene promotes the establishment and the circulation of microorganisms between animals of the herd, especially when good management practices are not commonly used.

Clustering analysis of RAPD-PCR based on Jaccard similarity index revealed a bacterial population arranged as single branches or clustered into small clonal groups (Figure 1). Closer relatedness was especially observed among *E. coli* strains isolated from the same mastitic milk samples. The clonal groups 1.02 and 1.03 represented *E. coli* isolated from Farm 1, from two different cows. The same occurred with sample 4.22, which exhibited identical RAPD genotypes compared to samples 4.20 and 4.21, isolated from different cows. Identical genetic profiles were also observed between *E. coli* samples 7.31 and 7.32 (Farm 7) and 9.42 and 9.43 (Farm 9) isolated from different quarters of a single cow.

RAPD clustering was not determined by the presence of substrates favoring the development of stable flies, feeding after milking and biochemical characteristics. Considering the clinical status, *E. coli* isolates obtained from the milk of cows with sub-clinical mastitis showed to be more closely related when compared with the *E. coli* from a cow with clinical mastitis. The overall chromosomal analysis of *E. coli* isolates from clinical and sub-clinical mastitis, revealed distinct genetic background which is consistent with previous observations with nonrelated epidemiological strains and thus characterizing distinct evolutionary lineages as suggested by Lamsey et al. (2013). On the other hand, different patterns also was described by Ramasoot et al. (2000) between clinical samples and subclinical each other's.

E. coli isolates obtained from the fly parts showed a high diversity between electrophoretic profiles, suggesting the *E. coli* strains were acquired from the environment rather than clinical situations (Ahmad et al. 2007). It could be explained since the most genetically related samples were

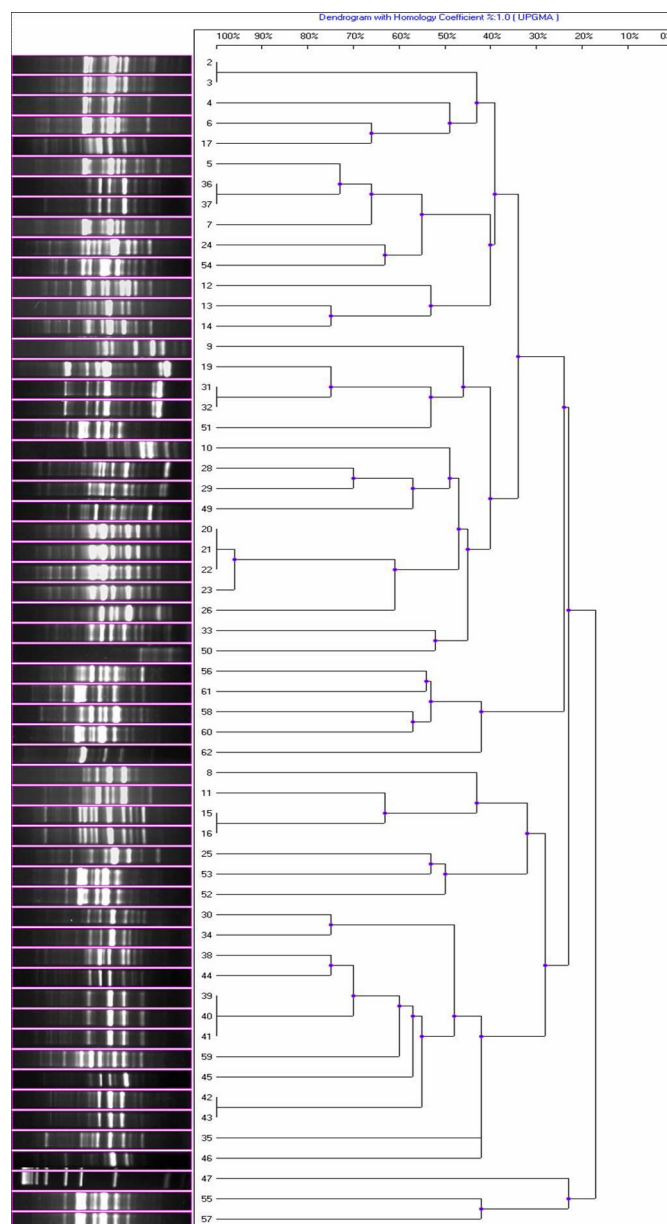


Fig.1. 1254 primer dendrogram profile of *Escherichia coli* samples from stable fly and mastitic milk.

detected at farms without any special characteristic other than poor milking hygiene.

The high genetic diversity detected in the bacterial population studied suggests that the *E. coli* isolates from milk and fly parts are not clonal related have a non-clonal origin. This diversity was expected, since when dispersed in the environment, the occurrence of genetic exchange between different bacteria population can be enhanced. These microorganisms are subjected to adverse conditions (Costa Sobrinho et al. 2012, Lopes et al. 2012) instead of particular epidemiological situations, as shown by the fact there were no shared profiles between farms or the municipalities visited.

This is the first study evaluating the genetic relationship of *E. coli* sub-populations isolated from mastitic milk and from flies. In similar studies, some authors have repor-

ted that muscidae are vectors of a determined pathogenic agent when similarity is observed in isolates from the stable fly and milk. However, there are no previous studies employing molecular tools to investigate genetic variability and clonal relationship of these sub-populations. Therefore, it is too early to state that the stable fly, or any other muscid, can be responsible for transmitting the bacteria in the environment (Moraes et al. 2004, Keen et al. 2006, Castro et al. 2007, Förster et al. 2009).

Our results highlight the possible role of the stable fly in spreading *E. coli* and reinforce the importance of controlling populations of this fly, in order to prevent the transmission of potentially pathogenic *E. coli* strains for humans and animals. This is of special concern, especially in farms where sanitation and training in animal management practices are required.

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