Bacterial antivirulence and evolution: significances for bacteriological diagnosis. A review

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SUMMARY

In last decades was initiated the concept of bacterial pathogen evolution from non-pathogenic ancestors by pathoadaptation by loss of gene function and it was defined an "antivirulence genes" as a gene whose expression in a bacterial pathogen is incompatible with the virulence of the pathogen. This article reviews briefly the examples of pathoadaptive mutation by loss of antivirulence genes in human and animal pathogens as *Shigella* spp., *Escherichia coli, Salmonella* spp., *Yersinia* spp., *Francisella tularensis* and *Burkholderia* spp., and the case of inactive *E. coli* strains, some of them enterotoxigenic, isolated from suckling pigs with diarrhoeic syndrome. Among Gram positive bacteria, the *Clostridium* genus may be interesting because some of the most pathogen species are practically non-fermentatives and some of the non-pathogen species are fermentatives, and thus the presence of antivirulence genes in *Clostridium* spp. is possible. In the laboratory diagnostics practice, the isolation of bacterial strains with atypical biochemical characters, especially inactive, may be a sign for loss of antivirulence genes and for presence of virulence characters.

Keywords: antivirulence genes, bacterial virulence, bacterial evolution, bacteriological diagnosis

INTRODUCTION

In bacteriological diagnostics activity at suckling piglets with diarrhoeic syndrome was sometimes isolated bacterial strains with atypical biochemical characters for his taxonomy framing. However, some of these atypical strains was virulent, so they was probably involved in the breakout of the diarrhoeic syndrome in suckling piglets (Sorescu et al., 2000). As the diarrhoeic syndrome implies highly morbidity, highly mortality and antibiotic treatments, and a fast and accurate diagnosis is helpful in limiting these damage and economic costs,

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is important to find a link between atypical biochemical characters and bacterial virulence of bacterial strains. There exist scanty literature data on the virulence of strictly this strains type. However, an explication may come from the concepts of antivirulence and bacterial pathogen evolution from non-pathogenic ancestors by pathoadaptation by loss of gene function, first described at human bacterial pathogens (*Shigella* spp.) and after at human and animal bacterial pathogens: *Salmonella* spp., *Yersinia* spp., *Francisella tularensis* and *Burkholderia* spp. (Maurelli, 2007; Maurelli et al. 1984; Bliven and Maurelli, 2012). Thus, this article reviews briefly the examples of pathoadaptive mutation by loss of antivirulence genes in human and animal bacterial pathogens and suggests bringing and using the results from bacterial antivirulence and evolution research to bacteriological diagnosis activity in animal breeding.

Material and Methods

A bibliographic review was performed on articles published in the last 26 years. Only articles focusing on bacterial pathogen evolution from non-pathogenic ancestors by pathoadaptation by loss of gene function and the bibliography correlate with inactive *E. coli* strains and *Clostridium* spp. identification biochemical characters were retained for this review.

Results and discussion

Bacterial strains with atypical biochemical characters are, some-times, isolated in the bacteriological diagnostics activity. That is the case for some inactive *E. coli* strains (Holt et al., 1994), isolated from the intestinal content of suckling piglets with diarrhoeic syndrome (Sorescu et al., 2000). These strains are immobile, do not produce gas through glucose fermentation, are lysine decarboxilase-negative and ferment lactose late (Table 1). Interestingly, from these five strains of inactive, Sereny's test negative (non-enteroinvasive) *E. coli* strains isolated, two are enterotoxigenic (thermolabile –LT- enterotoxine synthesisers) (Table 2) and they may be involved in the diarrhoeic syndrome breakout in suckling piglets (Sorescu et al., 2000). The strains differ from the atypic, virulent *E. coli* strains described in the literature (Le Minor and Richard, 1993) inasmuch as they do not belong to the enteroinvasive *E.coli* type (*EIEC*), but to the enterotoxigenic (*ETEC*) one. There wasn't known, at that time, other data about correlations between biochemical characters and virulence of inactive *E. coli* strains.

Table 1. Biochemical characters of five strains of inactive *E. coli* isolated from the intestinal content of suckling piglets with diarrhoeic syndrome (Sorescu et al., 2000).

Biochemical characters of inactive *E. coli* strains	Reaction
Glucose fermentation	+
Glucose fermentation with gas release	-
Lactose fermentation	
-48 hours/Triple Sugar Iron	-
-4 days/peptone water with 1% lactose	-
-5-7 days/peptone water with 1% lactose	+
Mannitol fermentation	+
Inositol fermentation	-
Sorbitol fermentation	+
Rhamnose fermentation	+
Sucrose fermentation	-
Melibiose fermentation	+
Amigdalin fermentation	-
Arabinosis fermentation	+
H_2S production	-
Indol production	+
Urease	-
Citrate use	-
Lysine decarboxilase	-
Phenylalanine desaminase	-
β-galactosidase	+
Arginine dehydrolase	-
Ornithine decarboxilase	-
Tryptophan desaminase	-
Acetoin production	-
Gelatinase	-
Cytochrome oxidase	-
Nitrate production	+

Symbols: +, reaction positive for all strains; -, reaction negative for all strains.

Table 2. Pathogenicity characters of five strains of inactive *E. coli* isolated from the intestinal content of suckling piglets with diarrhoeic syndrome (Sorescu et al., 2000).

Pathogenicity characters of inactive *E. coli* strains	Positive number of strains/ total number of tested strains
Thermolabile enterotoxine (LT)	2/5
Thermostabile enterotoxine (ST)	0/5
Sereny's test (enteroinvasiveness)	0/5
Shiga-like toxin (verotoxin)	0/3

Through his research started in 1980's with human bacterial pathogens, Maurelli (Maurelli, 2007; Maurelli et al. 1984; Bliven and Maurelli, 2012)

initiated the concept of bacterial pathogen evolution from non-pathogenic ancestors by pathoadaptation by loss of gene function, even if the evolution of bacterial pathogens is marked principally by the acquisition of virulence gene clusters. The genes that are no longer compatible with novel lifestyle of the pathogen are selectively inactivated and these genes are called “antivirulence genes”. Moreover, Maurelli (2007) supplementary defines an “antivirulence gene” a gene whose expression in a bacterial pathogen is incompatible with the virulence of the pathogen. The inactivation of ancestor’s antivirulence genes leads to a pathogen that is highly adapted to its host niche. In fact, the antivirulence genes function loss can be the result from bacterial adaptation to a new ecological niche (the macro-organism host with it defence mechanisms) and from necessity for optimize the energetic microorganism’s fitness through loss of the metabolic genes which become unnecessary for surviving in new environment. The antivirulence genes are involved in the bacterial metabolism, biofilm synthesis, lipopolisaccharides modification and host vasoconstriction (Bliven and Maurelli, 2012).

The first and best example of pathoadaptive mutation by loss of antivirulence genes is the case of the *cad A* gene in *Shigella* spp. and *E. coli.* While *Shigella* and *E. coli* share many biochemical characters, some properties differentiate *Shigella* from *E. coli*. So, the lysine decarboxilase (LDC) activity, encoded by the *cad A* gene in *E. coli*, is not expressed in *Shigella* and the *EIEC* strains -that cause a disease similar to dysentery caused by *Shigella* (Silva et al., 1980). The absence of LDC activity in the *Shigella* suggested that *cad A* may be an antivirulence gene for this species. The inhibitor of the virulence plasmid-encoded *Shigella* enterotoxin proved to be cadaverine, the product of the decarboxylation of lysine. As attenuation of virulence phenotypes is linked to expression of LDC (and production of cadaverine) in an *S. flexneri* 2a strain transformed with the *cadA* gene from *E. coli* K12, *cad A* has the properties of an antivirulence gene for *Shigella* (Maurelli, 2007). PMN transepithelial migration and phagolysosome escape are inhibited by *cad A*, also (Day et al., 2001; Fernandez et al., 2001; Maurelli et al. 1998). Interestingly, the inactive *E. coli* strains isolated by Sorescu et al., 2000, from suckling pigs with diarrhoeic syndrome also lack LDC activity, but some of them are enterotoxigenic (LT positive, so *ETEC*), which hints that these can be strains that make evolutionary pass from *E. coli* to *Shigella* spp.

E. coli and *Salmonella* diverged from a common ancestor approximately 100 million years ago (Doolittle et al., 1996). *E. coli* ferment lactose while *Salmonella* is traditionally thought of as a nonfermenter. In *E. coli* the *lac* system contains four genes, three of which are located in an operon (*lacZ, lacY* and *lacA*) and the fourth, *lacI*, encodes the *lac* operon repressor and negatively regulates the system under lactose-depleted conditions. *LacI* is an

antivirulence gene of *Salmonella* because the experimental expression of *lacI* into *Salmonella* decreased bacterial virulence (interferes with postinvasion events and induces downregulation of SPI-2 genes, which are critical in the assembly of the *Salmonella*-containing vacuole) (Eswarappa et al., 2009).

Loss-of-function mutations have played a role in the evolution of *Yersinia pestis* (the agent of bubonic and pneumonic plague, so, a strong pathogen in humans) from *Y. pseudotuberculosis* (a much milder enteric pathogen in humans) (Bliven and Maurelli, 2012). Approximately 200 genes are inactivated in *Y. pestis*, according to a genome comparasion with *Y. pseudotuberculosis* (Chain et al., 2004). *Y. pestis* lost antivirulence genes whose products repress biofilm synthesis (*rcsA*) and enhance biofilm degradation (*nghA*), and also lost *lpxL*, which encodes an acyltransferase that modifies bacterial LPS, leading to bacteria that are unable to stimulate host Tool-like receptor 4, which plays a critical role in pathogen invasion of the host immune response.

Francisella tularensis subsp. *tularensis* infects a broad mammalian host range and in humans induces a potentially fatal ulceroglandular or pneumonic tularemia. *F. tularensis* subsp. *holarctica* is only moderately virulent but both of them have lost the *pepO* gene, which is present at *F. tularensis* subsp *novicida*, a less virulent subspecia, typically occurring only as human pathogen in immunocompromised individuals. PepO is an M13 zinc metalloprotease and the loss of it allows bacterial dissemination (inhibits host vasoconstriction and permits the spread of the bacteria to systemic sites) and increased pathogenicity (Hager et al., 2006). PepO proteins from other bacterial species (*Streptococcus* and *Porphyromonas*) mimic the activity of the host vasoconstrictor and may represent lateral gene transfer between eukaryotes and prokaryotes (Awano et al., 1999; Froeliger et al., 1999). So, *pepO* is, also, an antivirulent gene, for *F. tularensis* subsp. *tularensis* and *holarctica* (Bliven and Maurelli, 2012).

Burkholderia mallei is an equine and human pathogen that causes a fatal systemic disease through infection of mucosal membranes. It evolved from *B. pseudomallei*, a soil organism that is capable of causing an opportunistic and localized infection in animal hosts (Woods et al., 1999; Wood, 2002). *B. pseudomallei* is believed to share a common ancestor with *B. thailandensis*, a non-pathogenic soil saprophyte. It is likely that loss of arabinose utilisation genes, from *B. thailandensis* (where this operon is encoded) to *B. pseudomallei* and *B. mallei* (where is absent), is also a pathoadaptative mutation in *B. mallei* (Maurelli, 2007). Thus, *araA*-*araH* antivirulence genes inhibit *B. pseudomallei* virulence in golden Syrian hamster model (Moore et al., 2004).

Table 3. Biochemical characters of some of the most pathogen species of *Clostridium* genus (Logan and De Vos, 2009; Stoica and Sorescu, 2016).

	C. tetani	*C. botulinum* type G	*C. novyi* type B	*C. difficile*
Lipase produced	-	-	-	-
Esculin hydrolysed	-	-	-	+
Starch hydrolysed	-	-	-	-
Nitrate reduced	-	-	d	-
Substrate utilized and/or acid produced from:				
Amygdalin	-	-	-	-
Arabinose	-	-	-	-
Cellobiose	-	-	-	+w
Fructose	-	-	d	+
Galactose	-	-	-	-
Glucose	-	-	+	+
Glycerol	nd	-	-	-
Glycogen	-	-	-	-
Inositol	-	-	-/+	-
Inulin	-	-	-	-
Lactose	-	-	-	-
Maltose	-	-	d	-
Mannitol	-	-	-	±
Mannose	-	-	+w	±
Melezitose	-	-	-	d
Melibiose	-	-	-	-
Raffinose	-	-	-	-
Rhamnose	-	-	-	-
Ribose	-	-	dw	-
Salicin	-	-	-	dw
Sorbitol	-	-	-	dw
Starch	-	-	-	-
Sucrose	-	-	-	-
Trehalose	-	-	-	dw
Xylose	-	-	-	dw

Symbols: +, reaction positive for 90-100% of strain; -, reaction negative for 90-100% of strains; d, 40-60% of strains positive; -/+, 11-39% of strains positive; ±, 61-89% of strain positive; w, weak.

From these researches (especially Sorescu et al., 2000; Maurelli, 2007; Bliven and Maurelli, 2012) it results that a strong pathogen bacterial species, well adapted to survive in host, is probably less biochemically active. Interestingly, upon author knowledge, all previous examples are about Gram negative bacteria. Among Gram positive bacteria, the *Clostridium* genus may be interesting because some of the most pathogen (*C. tetani, C. botulinum*

type G, *C. novyi* type B, *C. difficile*) species are practically non-fermentative (Table 3) and some of the non-pathogen species (*C. butiricum, C. estertheticum, C. lacusfryxellense*) are fermentative (Table 4). Consequently, the presence of antivirulence genes in *Clostridium* genus is entirely possible, all the more so there are some 'intermediate' species (fermentative and pathogen species and non-fermentative and less- or non-pathogen species, Table 5).

Table 4. Biochemical characters of some of the non-pathogen species of *Clostridium* genus (Logan and De Vos, 2009; Stoica and Sorescu, 2016).

	C. butyricum	*C. estertheticum*	*C. lacusfryxellense*
Lipase produced	-	+	NT
Esculin hydrolysed	+	-	NT
Starch hydrolysed	+	+	-
Nitrate reduced	-	+	NT
Substrate utilized and/or acid produced from:			
Amygdalin	±	-	+
Arabinose	±	d	-
Cellobiose	+	d	+
Fructose	+	+	NT
Galactose	+	+	+
Glucose	+	+	+
Glycerol	d	NT	NT
Glycogen	+	d	+
Inositol	d	+	+
Inulin	-/+	d	+
Lactose	+	-	+
Maltose	+	+	-
Mannitol	-/+	+	+
Mannose	+	+	-
Melezitose	-/+	-	+
Melibiose	+	+	+
Raffinose	+	+	+
Rhamnose	-	+	-
Ribose	+	-	+
Salicin	+	+	+
Sorbitol	-	+	-
Starch	+	+	+
Sucrose	+	+	+
Trehalose	+	-	+
Xylose	+	d	+

Symbols: +, reaction positive for 90-100% of strain; -, reaction negative for 90-100% of strains; d, 40-60% of strains positive; -/+, 11-39% of strains positive; ±, 61-89% of strain positive; w, weak; NT, not tested.

Table 5. Biochemical characters of 'intermediate' species of *Clostridium* genus (Logan and De Vos, 2009; Stoica and Sorescu, 2016).

	Pathogen species		Non-pathogen species	
	C.perfringens	*C.septicum*	*C.papyrosolvens*	*C.putrifaciens*
Lipase produced	-	-	-	-
Esculin hydrolysed	d	+	+	-
Starch hydrolysed	±	-	-	-
Nitrate reduced	±	d	-	-
Substrate utilized and/or acid produced from:				
Amygdalin	-w	-	-	-
Arabinose	-	-	+	-
Cellobiose	-/+	+w	+	-
Fructose	+	+	±	-
Galactose	+w	+w	+	-
Glucose	+	+	+	+
Glycerol	dw	-	+	NT
Glycogen	d	-	-	-
Inositol	±	-	NT	-
Inulin	-w	-	-	-
Lactose	+	+	-	-
Maltose	+	+	-	-
Mannitol	-	-	-	-
Mannose	+	+	-	-
Melezitose	-	-	-	-
Melibiose	-w	-	-	-
Raffinose	d	-	-	-
Rhamnose	-	-	-	-
Ribose	d	d	+	-
Salicin	-	d	-	-
Sorbitol	-/+	-	-	-
Starch	d	-	-	-
Sucrose	+	-	-	-
Trehalose	d	+w	-	-
Xylose	-	-	+	-

Symbols: +, reaction positive for 90-100% of strain; -, reaction negative for 90-100% of strains; d, 40-60% of strains positive; -/+, 11-39% of strains positive; ±, 61-89% of strain positive; w, weak; NT, not tested .

Thus, the non-fermentative character of strains of *Clostridium* spp., isolated from diseased animals, may be a sign for loss of antivirulence genes and for presence of virulence characters. These strains may be primarily involved in breakout of the respective disease and, even those from 'intermediate' species, must be carefully investigated for virulence factors, for

an accurate and efficient diagnosis. A future research can be made to find out the possible links between non-fermentative character of strains of *Clostridium* spp., especially those from 'intermediate' species, isolated from diseased animals, and the presence of virulence factors.

It is commonly agreed that antivirulence genes investigation supported the progress of understanding the bacterial pathogens evolution and, in the future, the study of antivirulence may help the discovery of novel medicinal products and vaccines (Blived and Maurelli, 2012).

CONCLUSIONS

This article reviews briefly the examples of pathoadaptive mutation by loss of antivirulence genes in human and animal pathogens, and the case of inactive *E. coli* strains, some of them enterotoxigenic, isolated from suckling pigs with diarrhoeic syndrome, and suggest a bring and use of the results from bacterial antivirulence and evolution research to bacteriological diagnosis activity in animal breeding. Among Gram positive bacteria, the *Clostridium* genus may be interesting because some of the most pathogen species are practically non-fermentatives and some of the non-pathogen species are fermentatives, thus the presence of antivirulence genes in *Clostridium* spp. is possible and may be investigated in a future research.

In this sense of a practical approach, in the laboratory diagnostics practice for animal breeding, the isolation of bacterial strains with atypical biochemical characters, especially inactive, may be a sign for loss of antivirulence genes and for presence of virulence characters. In this situation we strong recommend a broad investigation of virulence factors for an accurate and efficient bacteriological diagnosis since, usually, this kind of biochemical atypical strains are eliminated from supplementary and virulence tests

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REFERENCES

Awano, S., Ansai, T., Mochizuki, H., Yu, W., Tanzawa, K., Turner, A.J., Takehara, T., 1999. Sequencing, expression and biochemical characterization of the *Porphyromonas gingivalis pepO* gene encoding a protein homologous to human endothelin-converting enzyme. FEBS Letter 460:139-144.

Bliven, K.A. and Maurelli, A.T., 2012. Antivirulence genes: insights into pathogen evolution through gene loss. Infect. Immun. 80, 4061-4070.

Chain, P.S., Carniel, E., Larimer, F.W., Lamerdin, J., Stoutland, P.O., Regala, W.M., Georgescu, A.M., Vergez, L.M., Land, M.L., Motin, V.L., Brubaker, R.R., Fowler, J., Hinnebusch, J., Marceau, M., Medigue, C., Simonet, M., Chenal-Francisque, V., Souza, B., Dacheux, D., Elliott, J.M., Derbise, A., Hauser, L.J., and Garcia, E., 2004. Insights into the evolution of *Yersinia pestis* through whole-genome comparison with *Yersinia pseudotuberculosis*. Proc. Natl. Acad. Sci. U.S.A. 101:13826-13831.

Day, W.A. Jr., Fernandéz, R.E., Maurelli A.T., 2001. Pathoadaptive mutations that enhance virulence: genetic organization of the *cadA* regions of *Shigella* spp. Infect. Immun. 69:7471-7480.

Doolitle, R.F., Feng, D.F., Tsang S., Cho, G., Little, E., 1996. Determining divergence times of the major kingdoms of living organisms with a protein clock. Science 271, 470-477.

Eswarappa, S.M., Karnam, G., Nagarajan, A.G., Chakraborty, S., Chakrovortty D., 2009. *lac* repressor is an antivirulence factor of *Salmonella enterica*: its role in the evolution of virulence in *Salmonella*. PLoS One 4:e5789. doi:10.1371/journal.pone.0005789.

Fernandez, I.M., Silva, M., Schuch, R., Walker, W.A., Siber, A.M., Maurelli, A.T., and McCormick, B.A., 2001. Cadaverine prevents the escape of *Shigella flexneri* from the phagolysosome: a connection between bacterial dissemination and neutrophil transepithelial signalling. J. Infect. Dis. 184: 743-753.

Froeliger, E.H., Oetjien, J., Bond, J.P., Fives-Taylor, P., 1999. *Streptococcus parasanguis pepO* encodes an endopeptidase with structure and activity similar to those of enzymes that modulate peptide receptor signalling in eukaryotic cells. Infect. Immun. 67:5206-5214.

Hager, A.J., Bolton, D.L., Pelletier, M.R., Brittnacher, M.J., Gallagher, L.A., Kaul, R., Skerrett, S.J., Miller, S.I. and Guina, T., 2006. Type IV pili-mediated secretion modulates Francisella virulence. Mol. Microbiol. 62:227-237.

Holt, J.G., Krieg, N.R., Sneath, P.H.A., Staley, J.T., Williams, S.T. (Eds.), 1994. Bergey's Manual of Determinative Bacteriology, Ninth edition, Williams & Wilkins, Baltimore, pp. 175-222.

Le Minor, L., Richard, C., 1993. Méthodes de laboratoire pour l'identification des entérobactéries. Institut Pasteur, Paris, pp.55-72.

Logan, N.A. and De Vos, P., 2009. Genus I. Clostridium Prazmowski 1880, in: Vos, P.D., Garrity, G., Jones, D., Krieg, N.R., Ludwig, W., Rainey, F.A., Schleifer, K.-H., Whitman, W.B. (Eds.), Bergey's Manual of Systematic Bacteriology, Volume 3: The Firmicutes. Springer Dordrecht Heidelberg London New York, pp. 738-828.

Maurelli, A.T., 2007. Black holes, antivirulence genes, and gene inactivation in the evolution of bacterial pathogens. FEMS Microbiol. Lett. 267, 1-8.

Maurelli, A.T., Blackmon, B., and Curtiss, R. III, 1984. Temperature-dependent expression of virulence genes in *Shigella* species. Infect. Immun. 43:195-201.

Maurelli, A.T., Fernandéz, R.E., Bloch, C.A., Rode, C.K., Fasano, A., 1998. "Black holes" and bacterial pathogenicity: a large genomic deletion that enhances the virulence of *Shigella* spp. and enteroinvasive *Escherichia coli.* Proc. Natl. Acad. Sci. U.S.A. 95: 3943-3948.

Moore, R.A., Reckseidler-Zenteno, S., Kim, H., Nierman, W., Yu, Y., Tuanyok, A., Warawa, J., DeShazer, D., and Woods, D.E., 2004. Contribution of gene loss to the pathogenic evolution of *Burkholderia pseudomallei* and *Burkholderia mallei*. Infect. Immun. 72:4172-4187.

Silva, R.M., Toledo, M.R., and Trabulsi, L.R., 1980. Biochemical and cultural characteristics of invasive *Escherichia coli*. J. Clin. Microbiol. 11: 441-444.

Sorescu, I., Caraivan, I., Popa, V., Turcu, D., Tatu-Chitoiu, D., Tonciu, M., Tamas, A.I., Panca, C., Stanuica, D., Vlad, E., 2000. Characterisation of inactive *Escherichia coli* isolates from suckling pigs with diarrhoeic syndrome. Stud. Res. Vet. Med. (Bucharest), 8: 63-68.

Stoica, C., and Sorescu, I. *Regnum Prokariotae*, http//www.tgw1916.net, accessed on April 2016.

Woods, D.E., 2002. The use of animal infection models to study the pathogenesis of melioidosis and glanders. *Trends Microbiol.* 10: 483-484.

Woods, D.E., DeShazer, D., Moore, R.A, Brett, P.J., Burtnick, M.N., Reckseidler, S.L., and Senkiw, M.D., 1999. Current studies on the pathogenesis of melioidosis. *Microbes Infect.* 1:157-162.