CATÓLICA

UNIVERSIDADE CATÓLICA PORTUGUESA | PORTO

DETECTION OF *Pasteurellaceae* IN LABORATORY MICE BY FECAL PCR

Thesis presented to *Escola Superior de Biotecnologia* of the *Universidade Católica Portuguesa* to fulfill the requirements of Master of Science degree in Microbiology

by

Tânia Sofia Aguiar Ribeiro

under the supervision of

Isabel Fidalgo-Carvalho

Marta Vaz Mendes

September 2011

Resumo

A utilização de animais de laboratório microbiologicamente definidos na investigação biomédica tem-se tornado prática comum nas últimas décadas. A uniformização microbiológica, com base na realização de testes de rotina aos animais em intervalos regulares, tem contribuído para a política dos 3Rs (*Refinement*, *Reduction* e *Replacement*). Permite a redução do número de animais utilizados na medida em que diminui a variação dentro e entre grupos experimentais, e desempenha um papel importante no refinamento do estado de saúde dos animais melhorando assim o seu bem-estar. Adicionalmente, tem reduzido os riscos para a saúde humana devido a zoonoses. O estatuto sanitário SPF (*Specific Pathogen Free*) foi desenvolvido para garantir a ausência de patogénicos específicos em animais de laboratório enquanto o estatuto sanitário SOPF (*Specific Opportunistic and Pathogen Free*) garante a ausência dos principais agentes oportunistas para além dos patogénicos específicos. Estas definições têm base numa detalhada lista de exclusão de agentes susceptíveis de infectar roedores e confundir resultados de experiências com animais, tal como os membros da família *Pasteurellaceae*. Entre estes, a *Pasteurella pneumotropica* é considerada o patogénico oportunista mais frequente e é geralmente isolada do tracto respiratório superior, pulmões, tracto genital e gastrointestinal. A patogenicidade deste organismo em ratos e murganhos imunocompetentes é considerada baixa, mas em animais imunodeprimidos pode levar ao desenvolvimento de pneumonia, conjuntivite e infecção dos tractos respiratório e genital. Contudo, graças à estrutura taxonómica irresoluta da família *Pasteurellaceae* e à ocorrência de outros taxa para além da *P. pneumotropica*, a FELASA recomenda a monitorização de roedores SPF para todos os membros desta família. As técnicas de diagnóstico disponíveis para a detecção de *Pasteurellaceae* incluem métodos bacteriológicos e caracterização imunológica e bioquímica. Estes procedimentos são morosos e por vezes produzem resultados indeterminados dada a diversidade fenotípica desta família bacteriana. Ensaios de PCR com base na sequência do gene 16S rRNA foram recentemente descritos como alternativas para a detecção de *Pasteurellaceae*. No entanto, os protocolos utilizados baseiam-se em métodos de amostragem invasivos que requerem o sacrifício dos animais. Neste estudo desenvolvemos um ensaio de PCR simples, não invasivo e específico para a detecção de *Pasteurellaceae* usando DNA isolado de fezes de murganhos. Discutimos ainda o impacto desta técnica não invasiva na avaliação da prevalência de *Pasteurellaceae* em roedores de laboratório.

Abstract

The use of microbiologically defined laboratory animals in biomedical research has become standard practice in the last few decades. Microbiological standardization, based upon routine testing of the animals at regular intervals, has contributed to the 3Rs policy (Refinement, Reduction and Replacement). It allows the reduction of the number of animals used as it decreases the variation within and between test groups, and it plays an important role in the refinement of the overall health of laboratory animals thus improving their welfare. Additionally, it has reduced human health risks due to zoonotic diseases. Specific pathogen free (SPF) health status was developed to guarantee the absence of specific pathogens in laboratory animals whereas Specific Opportunistic and Pathogen-Free (SOPF) health status guarantees the absence of the major interfering opportunistic agents in addition to the specific pathogens. These negative definitions are based on a detailed exclusion list of agents which are likely to infect laboratory rodents and confound results from animal experiments, such as members of the *Pasteurellaceae* family. Among these, *Pasteurella pneumotropica* is considered to be the most frequently occurring opportunistic pathogen in laboratory rodents and it is usually isolated from the upper respiratory tract, lungs, genital and gastrointestinal tracts. The pathogenicity of this organism in immunocompetent laboratory mice and rats is regarded as low, but in immunodeficient animals it may lead to pneumonia, conjunctivitis, and respiratory and genital tract infections. However, due to the unsettled taxonomic structure of the *Pasteurellaceae* family and the occurrence of taxa other than *Pasteurella pneumotropica*, FELASA recommends the monitoring of SPF rodents for all *Pasteurellaceae*. The available diagnostic techniques for *Pasteurellaceae* screening traditionally include bacteriological methods, immunological and biochemical characterization. These procedures are time-consuming and sometimes yield indeterminate results due to the phenotypical diversity of this bacterial family. PCR assays based on the 16S rRNA gene sequence have recently been reported as alternatives to biochemical and culture methods for *Pasteurellaceae* detection. However, the protocols used are based on invasive sampling methods that require the sacrifice of animals. In this study we developed a simple, non invasive and specific PCR assay to detect *Pasteurellaceae* by using DNA isolated from mice feces. Furthermore we discuss the impact of this non-invasive technique in assessing the prevalence of *Pasteurellaceae* on laboratory rodents.

Acknowledgements

À Doutora Isabel Carvalho, minha orientadora, por esta tese. Muito obrigada pela disponibilidade, pelas críticas, sugestões e correcções durante a orientação deste trabalho. E sobretudo pela amizade, pelo carinho, pela motivação e pela confiança. Estou verdadeiramente grata por tudo o que tens feito por mim. Muito, muito obrigada!

À Doutora Marta Vaz Mendes, que prontamente aceitou a co-orientação deste trabalho, por ter sempre as portas abertas para mim. Por toda a ajuda indispensável, pela disponibilidade e motivação constantes.

À Sílvia Pires, pela ajuda preciosa na execução do trabalho prático relacionado com clonagem e sequenciação.

À Paula Magalhães pelos conselhos para as condições de PCR, e ao Francisco Morinha pela ajuda nos géis e no tratamento das sequências. Aos dois, muito obrigada pela amizade, pela cumplicidade e pela motivação.

Aos meus amigos, sempre interessados e solícitos em ajudar-me, por perdoarem as minhas ausências ao longo destes meses de escrita. Obrigada por estarem sempre presentes.

À minha família, a grande força que me impulsiona em tudo o que faço. À minha mãe, a minha fonte de inspiração, e aos meus irmãos, os melhores do mundo! Obrigada pelo apoio incondicional e por estarem presentes em todos os momentos.

Index

List of Abbreviations

ATCC: American Type Culture Collection

BLASTn: Nucleotide Basic Local Alignment Search Tool

bp: base pairs

DNA: Deoxyribonucleic acid

ELISA: Enzyme Linked Immunosorbent Assay

EMBL-EBI: European Molecular Biology Laboratory - European Bioinformatics Institute

FELASA: Federation of European Laboratory Animal Science Associations

GC: Guanine-Cytosine

HEPA: High-Efficiency Particulate Air

IBMC: Instituto de Biologia Molecular e Celular

ICSP: International Committee on Systematics of Prokaryotes

IVC: Individually Ventilated Cage

LPSN: List of Prokaryotic Names Standing in Nomenclature

NAD: Nicotinamide Adenine Dinucleotide

NCBI: National Centre for Biotechnology Information

NCTC: National Collection of Type Cultures

PAH: *Pasteurella-Actinobacillus-Haemophilus*

PCR: Polymerase Chain Reaction

RNA: Ribonucleic acid

rRNA: Ribosomal RNA

RTX: Repeats in the structural ToXin

SOPF: Specific and Opportunistic Pathogen Free

SPF: Specific Pathogen Free

UV: Ultraviolet

1. Introduction

1.1. Microbiological standardization and health monitoring of laboratory rodents

The use of microbiologically defined laboratory animals in biomedical research has become standard practice in the last few decades. Several groups of microorganisms (viruses, mycoplasmas, bacteria, fungi and parasites) have been recognized as responsible for infections in rodents, most of which are latent and do not lead to overt clinical signs. Latent infections can challenge the validity of animal studies and lead to a decreased reproducibility. There are numerous examples of the influence of latent microorganisms on the inflammatory cascade, immune response, physiology and behavior of laboratory animals. Indeed, all infections, apparent or unapparent, are likely to affect animal health and/or welfare and consequently increase variability on the results of animal experiments resulting in an increase in animal use. Importantly, some of the microorganisms that affect laboratory animals can also infect humans making animal health monitoring programs crucial for occupational health and safety in animal based research.

Standardized animals are important prerequisites for reproducible animal experiments. Microbiological standardization aims to produce animals that meet pre-established requirements of microbiological quality and to aid in the maintenance of this quality during experiments (Nicklas *et al*., 2002; Nicklas, 2008). Furthermore, it allows the reduction of the number of animals used as it decreases the variation within and between test groups, and it plays a role in the refinement of the overall health of laboratory animals thus contributing to the 3Rs policy (Refinement, Replacement and Reduction). Additionally, it has reduced human health risks due to zoonotic diseases. Proper health monitoring is vitally important to the evaluation of the microbial status of laboratory rodent colonies and it is based on periodic routine assessment of resident animals via random screening or targeted testing of sentinel animals.

With the advent of barrier-facility production techniques, rodents free of unwanted microorganisms can be produced for use in biomedical research. Specific pathogen free (SPF) health status was developed to guarantee the absence of specific pathogens in laboratory animals whereas Specific Opportunistic and Pathogen-Free (SOPF) health status guarantees the absence of the major interfering opportunistic agents in addition to the specific pathogens.

These negative definitions are based on a detailed exclusion list and indicate that the rodents have been tested and found to be free of the pathogen and opportunistic agents designated in that exclusion list (Otto and Franklin, 2006).

Appendix 3 Health monitoring reports

Health Monitoring in Accordance with FELASA recommendations

Date of issue:

Location: Housing: (Barrier/Non-Barrier/IVC/Isolator):

Species: Mouse Strain: (Strain)

Species and strains present within the unit:

	Test frequency	Latest test date	Latest results	Testing laboratory	Test method	Historical results (≤ 18 months)
Viruses						
Mouse hepatitis virus	3 months					
Mouse rotavirus (EDIM)	3 months					
Parvoviruses						
Minute virus of mice	3 months					
Mouse parvovirus	3 months					
Pneumonia virus of mice	3 months					
Sendai virus	3 months					
Theiler's murine encephalomyelitis virus	3 months					
Ectromelia virus	Annually					
Lymphocytic choriomeningitis virus	Annually					
Mouse adenovirus type 1 (FL)	Annually					
Mouse adenovirus type 2 (K87)	Annually					
Mouse cytomegalovirus	Annually					
Reovirus type 3	Annually					
Additional organisms tested:						
Bacteria, mycoplasma and fungi						
Citrobacter rodentium	3 months					
Clostridium piliforme (Tyzzer's disease)	3 months					
Corynebacterium kutscheri	3 months					
Mycoplasma spp.	3 months					
Pasteurellaceae	3 months					
Salmonella spp.	3 months					
Streptococci β-haemolytic (not group D)	3 months					
Streptococcus pneumoniae	3 months					
Helicobacter spp.	Annually					
Streptobacillus moniliformis	Annually					
Additional organisms tested:						
Parasites						
Ectoparasites: Species designation	3 months					
Endoparasites: Species designation	3 months					
Pathological lesions observed	3 months					

Data are expressed as number positive/number tested

Positive findings in other species in the same unit:

Abbreviations used in this report:

ELISA = enzyme linked immunosorbent assay, MICR = microscopy, IFA = immunofluorescence assay, CULT = culture, PATH = gross pathology, PCR = polymerase chain reaction, HIST = histopathology, NT = not tested

Figure 1: Agents exclusion list recommended by FELASA (2002) for SPF mouse colonies.

To promote international harmonization, the Federation of European Laboratory Animal Science Associations (FELASA) has established an exclusion list (Figure 1) which is the most followed recommendation for rodents' health monitoring in European and non European animal facilities. *Pasteurellaceae* are present in the FELASA exclusion list for SPF rodent colonies. Whether they should be a part of this list remains quite controversial, but due to the absence of overt clinical signs and to their high prevalence in laboratory rodent colonies there is general consensus in including these agents in health monitoring programs. According to the FELASA recommendations for health monitoring of mouse and rat colonies, the presence of *Pasteurellaceae* should be monitored quarterly (Nicklas *et al*., 2002).

1.2. *Pasteurellaceae*

1.2.1. Taxonomy

The name *Pasteurellaceae* was established by Pohl in 1979 and validly published in 1981. DNA hybridization studies revealed a close relationship between *Haemophilus* spp, *Pasteurella* spp and *Actinobacillus* spp, leading to the establishment of the family *Pasteurellaceae* which was located in the gamma division of the *Proteobacteria* phylum (Mannheim, 1984). In 1992, the phylogenetic relationships in representative strains of some *Pasteurellaceae* species were assessed by comparison of 16S rRNA gene sequences (Dewhirst *et al*., 1992, 1993). Since then, the taxonomic classification of these fastidious bacteria has been under constant revision as a consequence of the increasing phylogenetic data obtained by molecular methods. Sequencing of the housekeeping genes *atpD*, *infB*, *rpoB* and *gyrB*, and of the 16S rRNA gene has redefined the relationships among the various species of the family, providing new insights to delineate the phylogeny of the *Pasteurellaceae* family (Christensen *et al*., 2004, 2007; Korczak *et al*., 2004; Hayashimoto *et al*., 2005b, 2006, 2007b; Sasaki *et al*., 2006a, 2006b, 2009a). However, the correct taxonomic position of these agents has not yet been defined.

According to the most recent taxonomy from the Subcommittee on the Taxonomy of *Pasteurellaceae* of the International Committee on Systematics of Prokaryotes (ICSP; Christensen, 2009) and to the List of Prokaryotic names Standing in Nomenclature (LPSN; Euzéby, 2011), the following 15 different genera have been described to date: *Actinobacillu*s, *Aggregatibacter*, *Avibacterium*, *Basfia*, *Bibersteinia*, *Chelanobacter*, *Gallibacterium*,

Haemophilus, *Histophilus*, *Lonepinella*, *Mannheimia*, *Nicoletella*, *Pasteurella*, *Phocoenobacter* and *Volucribacter*. *Bergey's Manual of Systematic Bacteriology* subdivides the *Pasteurellaceae* into 21 phylogenetic clusters. Only five clusters are represented by named genera, namely *Actinobacillus sensu stricto*, *Haemophilus sensu stricto*, *Pasteurella sensu stricto*, *Manheimia* and *Lonepinella*. The remaining 16 clusters contain a wide variety of hitherto unnamed species and strains, among which are strains misclassified as *Actinobacillus*, *Haemophilus* and *Pasteurella* (Olsen *et al.*, 2005).

1.2.2. General characteristics

Bacterial species belonging to the *Pasteurellaceae* family are Gram-negative, facultative anaerobic, coccoid- or rod shaped, non-spore-forming and nonmotile. The GC content of the DNA is 37-47% and the genome size range is between 1.7 and 2.6 Mb. They produce acid from glucose, usually without the production of gas. Nitrate reductase, oxidase, catalase and alkaline phosphatase tests are almost always positive, although the oxidase reaction may be weak or delayed. These species are routinely grown i*n vitro* under aerobic conditions, but an atmosphere enriched with 5 to 10% carbon dioxide usually improves growth. The optimum growth temperature is 35-37ºC and most strains grow within 24 to 48 hours of incubation. They are easily cultured in blood agar but most species also grow well on chocolate agar. Certain species are dependent on growth factors such as X-factor (protoporphyrin, hemin) or V-factor (β-NAD) (Nicklas, 2007; Hayashimoto *et al*., 2007b, 2008; Dousse *et al*., 2008).

The species of the *Pasteurellaceae* family generally colonize the mucosal membranes of the respiratory, digestive and genital tracts of mammals, birds and reptiles. They may also be recovered from secretions. The family includes both pathogenic and non-pathogenic species for many animals and comprises mostly commensal species. However, several *Pasteurellaceae* are known as opportunistic secondary invaders, able to cause infections under predisposing circumstances, and some species are primary pathogens that cause severe disease in many animals (Steffen and Nicklas, 1999; Olsen *et al*, 2005).

Few molecular studies have been undertaken and many strains and species infecting laboratory rodents have not been sufficiently characterized. Although members of the genera *Actinobacillus* and *Haemophilu*s have been reported in laboratory rodents (Csukas, 1975; Bisgaard, 1986), very little information has been published on these organisms. The genus *Pasteurella* includes the most well-known bacteria of this family found in mice (*Mus*

musculus) and rats (*Rattus norvegicus*). Among these, *Pasteurella pneumotropica* is considered to be the most frequently occurring opportunistic pathogen in contemporary conventional and barrier-maintained laboratory rodents (Nicklas, 2007; Pritchett-Corning *et al*., 2009).

1.3. *Pasteurella pneumotropica*

1.3.1. Taxonomy

Pasteurella pneumotropica was first described as a latent pneumotropic *Pasteurella* isolated from the lungs of laboratory mice (Jawetz, 1950). The biotype Heyl was proposed latter, based on the utilization of carbon sources and amino acids (Heyl, 1963), and *P. pneumotropica* was reclassified as *P. pneumotropica* biotypes Jawetz and Heyl. The third biotype, Henriksen, is not found in rodents and has been reclassified as *P. dagmatis* (Mutters *et al.*, 1985). Subsequently, the detailed biochemical variations of isolates of *Pasteurellaceae* obtained from laboratory and wild rodents were compared with those of reference strains, including *P. pneumotropica* Heyl ATCC 12555 (FJ685623) and *P. pneumotropica* Jawetz NCTC 8141 (AY362924) (Boot and Bisgaard, 1995c). Further, a report showed that *P. pneumotropica* isolates could de differentiated by haemagglutination properties (Boot *et al*., 1993).

In bacterial taxonomy, *P. pneumotropica* is closely related to *Actinobacillus muris* and *Haemophilus influenzaemurium* and it is commonly referred as belonging to the *Pasteurella-Actinobacillus-Haemophilus* (PAH) group. However, these pathogenic agents have not yet been formally classified under genera since there is insufficient knowledge regarding the taxonomic classification of these bacteria. Although many wild-type strains of *P. pneumotropica* have been isolated mainly from laboratory rodents, and their biochemical properties have been aligned, their phenotypic and genotypic characteristics have diversified with the increase of new isolates. Furthermore, details regarding the phylogeny of wild type strains of *P. pneumotropica* have not yet been clarified (Hayashimoto *et al*., 2005b; Sasaki *et al*., 2006a, 2006b). Strains reported as *P. pneumotropica* are genetically diverse and *Pasteurellaceae* from hamsters and guinea pigs belong to other taxa than those from mice and rats (Olsen *et al*., 2005). A report suggests the existence of *P. pneumotropica* isolates that can infect both mice and rats, whilst other isolates can only infect one of these hosts (Nakawaga *et*

al., 1981). Other reports suggest the existence of a host-independent group of isolates in addition to the host-dependent group (Sasaki *et al.*, 2006b, 2009a). Moreover, although *P. pneumotropica* is well known as a non-hemolytic bacterium, isolates from mice and rats showing β–hemolytic activity on blood agar were also reported (Sasaki *et al*., 2006a, 2006b). Various methods of detection and identification of *P. pneumotropica* in laboratory rodents have been proposed and developed in recent years, but the taxonomic differences in *P. pneumotropica* strains remain unelucidated (Sasaki *et al*., 2009a).

1.3.2. Organism description

P. pneumotropica is a Gram-negative, aerobic or facultative anaerobic, nonhemolytic, nonmotile and non-spore forming short rod or coccobacillus. On primary culture, the organism grows well on blood agar producing smooth, convex, light gray to yellow nonhemolytic colonies after 48 hours incubation at 37ºC. It produces acid but not gas from glucose. It is oxidase, urease and catalase positive (Steffen and Nicklas, 1999; Hayashimoto *et al*., 2005a; Nicklas *et al*., 2007).

There have been several opinions about the separation of the *P. pneumotropica* Jawetz and Heyl biotypes. However, reports in the literature on the biochemical and phenotypical characteristics of both biotypes are very inconsistent and contain conflicting information (Heyl, 1963; Kodjo *et al*., 1999; Nicklas, 2007).

Heyl and Jawetz biotypes are approximately 96% similar with respect to 16S rRNA gene Although there are data suggesting that the Heyl biotype is more pathogenic than Jawetz, correlations between a specific biotype and pathogenicity have not yet been firmly established (Hayashimoto *et al*., 2005a; Dole *et al*., 2010).

1.3.3. Hosts

P. pneumotropica is an opportunistic organism prevalent in many commercial and research colonies of rodents. It has been frequently isolated from both healthy and diseased laboratory rodents, notably from mice and rats, but also from hamsters and guinea pigs (Kunstyr and Hartmann, 1983; Nicklas *et al*, 2007). Surveys of conventional rodent colonies have revealed a very high incidence of asymptomatic infection with this organism.

Association of *P. pneumotropica* with disease in humans is exceedingly rare, but sporadic case-reports of human infections are found in literature, such as septicemia in a 72-year-old immunocompetent woman who was frequently scratched by cats and dogs (Frebourg *et al*., 2002) or peritonitis in a peritoneal dialysis 8-year old patient resulting from a contamination of the dialysis tube by a pet hamster bite (Campos *et al.*, 2000). Human pasteurellosis are usually associated with animal contact, and are most often caused by dog and cat bites resulting in cellulitis and subcutaneous abscesses. The second most common site of infection or colonization is the respiratory tract. Human systemic infection is very rare and mostly occurs in patients with underlying disease as respiratory chronic disease (Gautier *et al*., 2005; Guillard *et al*., 2009, 2010).

1.3.4. Basic biology

In laboratory rodent colonies, *P. pneumotropica* is most likely transmitted by direct contact between infected animals, but indirect exposure to contaminated bedding or other fomites may also occur (Sharmann and Heller, 2001). The organism does not appear to be vertically transmitted and it is not secreted in the milk. In neonates, the transmission occurs through intravaginal infection at the parturition, and oro-nasal infection through the maternal feces and saliva (Mikazuki *et al*., 1994). *P. pneumotropica* is ubiquitous in many rodent colonies and it can be detected in most mucous membranes of their hosts. Main colonization sites are the upper respiratory tract, lungs and genital tracts but they are also frequently isolated from the intestinal tract, feces, urinary bladder, oral cavity, conjunctiva and skin (Needham *et al*., 1975; Ward *et al*. 1978; Mikazuki *et al*., 1994; Wang *et al*., 1996; Goelz *et al*., 1996). The pharyngolarynx is the primary colonization site of *P. pneumotropica* in mice (Mikazuki *et al*., 1994).

1.3.5. Clinical disease and pathogenesis

In contemporary mice colonies, *P. pneumotropica* infection is usually subclinical but may be associated with sporadic disease, including conjunctivitis, panophthalmitis, respiratory genital and intestinal tract infections, metritis, cystitis, dermatitis, abscesses and suppurative lesions (Needham and Cooper, 1975; Ward *et al*., 1978; Nicklas, 2007). These manifestations

occur most commonly in immunodeficient or genetically modified mouse strains, in which *P. pneumotropica* infection may lead to various serious diseases such as lethal pneumonia and sepsis. It is known that coinfection with *Pneumocystis carinii* and *P. pneumotropica* leads to fatal pneumonia in B cell-deficient mice (Macy *et al*., 2000). In mice lacking functional alleles at MHCII, *Tlr4* and *Nramp1* genes, experimental challenge with *P. pneumotropica* results in lung infections and lethal pneumonia (Chapes *et al*., 2001; Hart *et al.,* 2003). Furthermore, orbital abscesses were caused by *P. pneumotropica* infection in Cd28-mutated mice (Artwohl *et al*., 2000). Historically, *P. pneumotropica* infection in laboratory rodents was associated with disease but only when concurrent infections with other pathogens were present, such as Sendai virus (Jakab and Dick, 1973; Carthew and Aldred, 1988), Kilham rat virus (Carthew and Gannon, 1981), *Pneumocystis carinii* (Macy *et al.*, 2000) or *Mycoplasma pulmonis* (Brennan *et al*., 1969; Laubach *et al*., 1978). Reports of *P. pneumotropica* as the sole cause of disease in immunocompetent mice are scarce, even when a large proportion of the mice colony is colonized with the bacterium.

P. pneumotropica pathogenesis has remained unknown because the virulence factors involved in the pathogenicity have not yet been thoroughly identified and characterized (Kawamoto *et al*., 2007). The phenotypic characteristics related to the virulence of *P. pneumotropica* are hemagglutination and hemolysis (Sasaki *et al*., 2009b). RTX toxins are considered to be important virulence factors in *Pasteurellaceae*. These pore-forming protein toxins are produced by a broad range of pathogenic gram-negative bacteria. *In vitro*, they mostly exhibit a cytotoxic and often also a hemolytic activity. They are particularly widespread in species of the *Pasteurellaceae* family which cause infectious diseases, most frequently in animals but also in humans (Frey and Kuhnert, 2002). In two recent studies, three hemolysin-like proteins similar to RTX toxin were identified and characterized in *P. pneumotropica* (Sasaki *et al*., 2009b, 2011). However, details about their functions and cytotoxicity remain to be clarified. More recently, a study suggest that RTX toxins may have a determinant role in host specificity of pathogenic species of *Pasteurellaceae* (Frey, 2011).

1.3.6. Treatment and elimination

P. pneumotropica infections in laboratory rodents can be effectively treated with antibiotics. Enrofloxacin, a fluoroquinolone bactericidal antibiotic, has been shown to be effective in eliminating this organism from mice colonies when administered by oral route

(Goelz *et al*., 1996; Ueno *et al*., 2002; Matsumya *et al*., 2003; Sasaki *et al*., 2007). Rederivation techniques such as hysterectomy and embryo transfer are known to be effective in eliminating *P. pneumotropica* (Goelz *et al*., 1996; Macy *et al.*, 2000). However, treatment and elimination procedures are time-consuming and require special facilities and equipment. Therefore, to prevent infection in laboratory rodents, it is necessary to periodically perform microbiological monitoring of the animals and maintain the rodent colonies in a clean environment.

1.4. Prevalence of *Pasteurellaceae* in laboratory rodents

Pasteurellaceae infections in laboratory rodents have a worldwide distribution. Most published reports mention only *P. pneumotropica*, but it has to be expected that all species are likely to be found in laboratory rodents in all parts of the world due to the extensive exchange of animals between research institutes. Historically, the number of rodent colonies infected with *P. pneumotropica* has been higher for conventional colonies but the agent has also been commonly found in barrier-maintained animals (Nakagawa *et al*., 1984). In a retrospective study from laboratory rodent colonies in France, more than 40% of mice and rat colonies were reported to be positive for *P. pneumotropica* (Zenner and Regnault; 1999/2000). Recently, in a microbiologic screen of 109.403 mice from North America and Europe, the prevalence of *P. pneumotropica* was found to be 12,9% (Pritchett-Corning *et al.*, 2009). Even today, *Pasteurellaceae* are frequently tolerated as "normal flora" and eradication steps are not taken, which is one reason for the high prevalence rate in laboratory rodents colonies.

1.5. Effects of *Pasteurellaceae* on animal based research

Members of the *Pasteurellaceae* family are prevalent in laboratory rodent colonies and frequently colonize the upper respiratory tract and other mucosal surfaces of rodents. The species of widest distribution and of major concern is undoubtedly *P. pneumotropica*, but its role in rodents' health monitoring remains quite controversial. Because clinical disease is infrequent in immunocompetent mice colonized with *P. pneumotropica*, this bacterium is often perceived as having little or no clinical relevance to most biomedical research studies (Ueno *et al*., 2002). However, infections with other agents, including mouse parvoviruses,

mouse rotavirus, and *Helicobacter spp.*, can alter host physiology or biologic responses without causing clinical signs of illness (Baker, 1998). A recent study showed that experimental inoculation of immunocompetent mice with *P. pneumotropica* can induce perturbations in the transcription of inflammatory cytokines (Patten *et al*., 2010).

The most common viewpoint regarding *P. pneumotropica* is that it is a major opportunistic pathogen or co-pathogen capable of contributing to serious disease by acting synergistically with other infectious agents. As such, it has the potential to confound interpretation of data collected from infected animals. Knowing the full health status of experimental mice is therefore paramount to avoid unwanted experimental variables. Although most laboratories or institutions report the presence or absence of *P. pneumotropica*, FELASA recommends that all *Pasteurellaceae* should be listed in laboratory rodents' health reports because the taxonomy of the *Pasteurellaceae* family is complex and at present incompletely resolved (Nicklas *et al*., 2002).

1.6. Detection of *Pasteurellaceae* in laboratory rodents

Pasteurellaceae infection in laboratory rodents can be monitored by standard microbiological culture and morphological examination, immunological and biochemical characterization, serologic assays and, more recently, molecular based diagnostic methods (Steffen and Nicklas, 1999; Nicklas *et al.*, 2002; Hayashimoto *et al.*, 2005a; Dousse *et al*., 2009).

1.6.1. Traditional methods

Although there is no standardized identification procedure of *Pasteurellaceae* in microbiological monitoring of laboratory rodents, isolation and identification using classical bacteriology techniques is still used as the "gold standard" in diagnostic laboratories. Several primary isolation media can be used although blood agar is common choice. However, the organisms are sometimes difficult to culture because they are present in small numbers or may be overgrown by other bacteria (Bootz *et al*., 1998). Furthermore, morphological examination is subjective and it has been shown repeatedly that different laboratories come to

different conclusions on the same strain of *Pasteurellaceae* (Hayashimoto *et al.*, 2005a; Nicklas *et al*., 2002).

Commercial systems for biochemical characterization, including API® 20 NE and Vitek® 2 systems (BioMérieux), are commonly used for *Pasteurellaceae* identification, but failure of these commercial systems to satisfactorily identify microorganisms is of concern. A case of *Pasteurella dagmatis* misidentified as *P. pneumotropica* in the automated system Vitek® 2 was reported because the system database included very few *Pasteurellaceae* species (Guillard *et al*., 2009). Furthermore, the FELASA Working Group on Health Monitoring of Rodent and Rabbit Colonies indicated that commercial kits do not identify *Pasteurellaceae* properly (Nicklas *et al*., 2002).

Several ELISA assays have been described for the detection of *Pasteurellaceae* and *P. pneumotropica* antibodies in the sera of laboratory rodents (Manning *et al.*, 1989; Boot *et al*., 1995a, 1995b; Boot and van den Berg, 2006). However, there is a high risk of false-positive reactions due to the complex antigenic structure of bacteria and to unpredictable cross-reactivities between different isolates. Moreover, owing to the diversity of *Pasteurellaceae*, several antigens are required to cover all the species in the family (Bootz *et al*., 1998; Nicklas *et al.*, 2002).

Molecular based techniques have been reported as alternatives to biochemical, serologic and culture methods as these procedures are time-consuming and sometimes yield indeterminate results due to the phenotypical diversity of the *Pasteurellaceae* family.

1.6.2. Molecular based methods

A number of Polymerase Chain Reaction (PCR) assays have been described to identify and biotype cultured isolates of *Pasteurellaceae* from laboratory rodents or for their detection in clinical samples. Target sequences for primers are usually located on the 16S rRNA gene (Wang *et al*., 1996; Bootz *et al*., 1998; Weigler *et al.*, 1998; Kodjo *et al*., 1999; Nozu et al., 1999; Hasegawa *et al.*, 2003) but assays targeting the *gyrB* and the *rpoB* genes have also been described (Hayashimoto *et al*, 2007a; Dole *et al*., 2010).

16S rRNA qualifies as the most comprehensive single gene database that can be used to classify bacteria phylogenetically and has emerged as the most prominent target in microbial detection. Although much of the 16S rRNA gene is highly conserved among many bacterial families, portions of the gene are unique and can be used to speciate bacteria (Dole *et al*.,

2010). Wang *et al.* (1996) established their PCR based on the 16S rRNA gene sequence of the type strain of *P. pneumotropica* Jawetz. However, due to the genetic heterogeneity of *P. pneumotropica*, their primer sets do not detect *P. pneumotropica* Heyl or other *Pasteurellaceae* infecting or colonizing rodents. Nozu *et al*. (1999) reported a PCR with new primers designed also from *P. pneumotropica* 16S rRNA gene sequences. A number of reference and field strains were used to evaluate the PCR, and differences were shown between their results and those obtained by Wang and colleagues (1996). However, neither the sensitivity nor the specificity can be evaluated on the basis of the given data since *P. pneumotropica* was identified by API® 20 NE which is not suited for this purpose. Kodjo *et al.* (1999) developed a PCR which detects *P. pneumotropica* biotypes Jawetz and Heyl. Bootz *et al.* (1998) selected primers on the basis of 16S rRNA gene sequences of various rodent isolates representing different phenotypic groups of *Pasteurellaceae* together with all sequences from NCBI GenBank and EMBL-EBI databases available at that time. This PCR was established with the goal of detecting all *Pasteurellaceae* known to colonize mice and rats.

The different PCR primer sets targeting the 16S rRNA gene described to date differ in their ability to detect *Pasteurellaceae* infection in rodents. According to a recent study, primer pairs based on the 16S rRNA gene sequence described by Wang *et al*. (1996) and Kodjo *et al*. (1999) should be considered unsuitable to monitor rodents for *Pasteurellaceae* infection as they will detect only a fraction of the taxa from the bacterial family cultured from rodents. The primer pair developed by Bootz *et al.* (1998) revealed to detect significantly more *Pasteurellaceae* than both the other pairs (Boot *et al*., 2009).

The success of detection assays based on PCR has been largely due to its rapidity in comparison to many conventional diagnostic methods. Additionally, there is the enormous potential of DNA amplification assays with regard to sensitivity and specificity.

1.6.3. Sampling techniques

A variety of samples have been used for *Pasteurellaceae* screening in laboratory rodents, both with traditional and molecular based methods. However, the sampling techniques described are frequently invasive and require the sacrifice of animals. Usually samples are obtained from tissue specimens or swabs, including pharynx, trachea and lung samples (Bootz *et al*., 1998; Chapes *et al*., 2001), oropharyngeal swabs (Ueno *et al*., 2002), swabs from

conjunctiva, nasal cavity, pharyngolarynx, vagina, uterus and cecum (Nozu *et al.*, 1999; Scharmann and Heller, 2001) or nasopharynx and reproductive tract specimens and swabs (Steffen and Nicklas, 1999; Kodjo *et al.*, 1999). The use of oral swabs from live animals has been described as an alternative non-invasive sampling method (Patten *et al.*, 2010). However, this method is technically demanding and it requires the restraint of animals causing a certain level of distress and discomfort. PCR assays using fecal pellets have been described for several rodent pathogens, including *Helicobacter spp.* (Beckwith *et al.*, 1997), *Clostridium piliforme* (Furukawa *et al.*, 2002) and *Citrobacter rodentium* (McKeel *et al.*, 2002). Fecal samples are easy to obtain and provide a good source of bacterial DNA. Therefore, they can be used as a non-invasive means of rapidly screening large numbers of animals. Furthermore, this sampling method follows the 3Rs policy by refining animal welfare, and by reducing the number of animals used for health monitoring purposes.

1.7. Work Objectives

Recently, immunodeficient animals have become important for experimental biomedicine. Therefore, managing pathogens that do not significantly affect the health of immunocompetent animals but cause severe diseases in immunodeficient and genetically modified animals has become a standard practice. Preventing the propagation of *P. pneumotropica* and monitoring infections are important issues in the management of laboratory rodents and, therefore, this bacterium is included as a routine test item for the microbiological monitoring of laboratory rodents. But due to the unsettled taxonomic structure of the *Pasteurellaceae* family and the occurrence of taxa other than *P. pneumotropica* in rodents, FELASA recommends the monitoring of rodents for all *Pasteurellaceae* (Nicklas *et al.*, 2002).

A simple, sensitive and non-invasive PCR assay to detect the presence of *Pasteurellaceae* would facilitate the detection of latent infections and would be especially valuable as a surveillance tool in large colonies of SPF rodents. Therefore, the purpose of this work was to,

- develop a novel fecal PCR assay for detection of *Pasteurellaceae* in laboratory mice;
- compare this assay with traditional bacteriological methods;
- discuss the impact of this non-invasive technique in assessing the prevalence of *Pasteurellaceae* on laboratory rodents.

2. Materials and Methods

2.1. Animals

This investigation was carried out using mice from the IBMC's Animal House internal health monitoring program. A total of 362 mice of 49 strains including knockout, transgenic and wild type mice of various genetic backgrounds, immune status, ages and either sex were examined. The animals used were breeders, experimental mice, stock mice or bedding sentinels. Except for 2 animals, kept in a non-barrier conventional room, all other 360 mice were kept in full-barrier rooms and their health status was considered to be SOPF for the agents listed in the FELASA recommendations for the monitoring of mice colonies (Nicklas *et al.*, 2002). All animals were kept in rooms under positive pressure HEPA-filtered ventilation, at 22 ± 2ºC with a 12/12h light/dark cycle and 55 ± 10% relative humidity, housed in static polycarbonate top filtered cages on corn cob bedding, either in shelf racks (Figure 2A) or in Individually Ventilated Cages (IVCs; Figure 2B). Autoclaved diet (ref. 4RF25GLP, Mucedola) and reverse-osmosis filtered water were available *ad libitum*. Cages and bedding, feeders and water bottles were autoclaved and changed every week. All procedures were carried out in accordance with the national law Portaria nº 1005/92 (23rd of October) and the European Communities Council Directive 86/609/EEC.

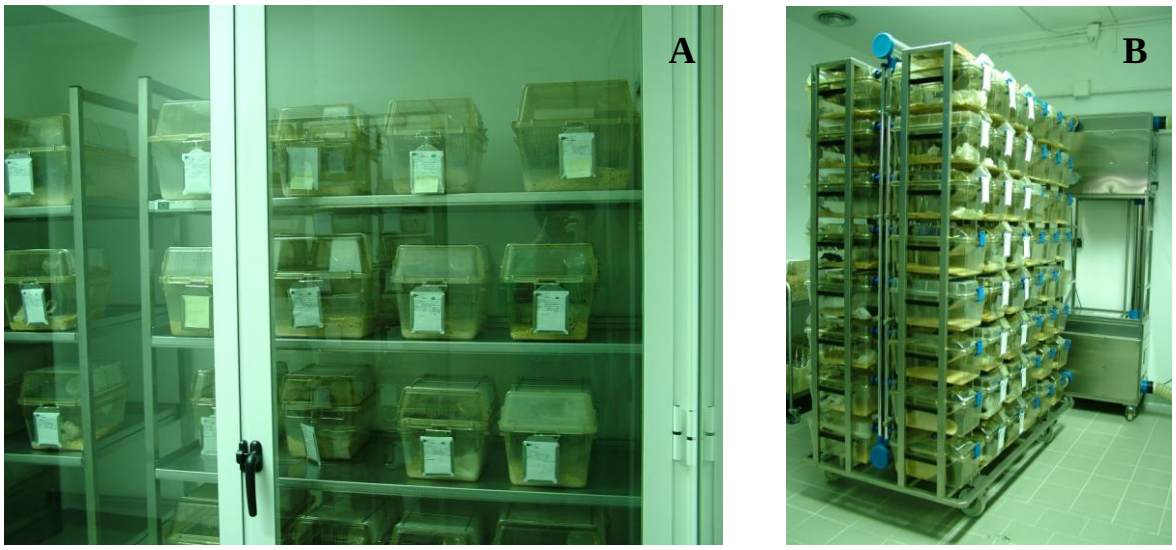


Figure 2: Animals housed in static polycarbonate top filtered cages. **A:** Shelf racks. **B:** IVC.

2.2. Sampling

For DNA isolation, stool samples were obtained by taking individual mice out of their cage and collecting a fecal pellet directly into a sterile 1.5 ml reaction tube by holding the mouse by the base of the tail (Figure 3). More than 90% of mice excreted a fecal pellet within one minute. After collection, samples were immediately frozen at -20ºC and DNA isolation was performed within 24 hours. For culture purposes, oral swabs (Becton, Dickinson and Company) were obtained by restraining each mouse for about 2 minutes while swabbing the animal's mouth. Swabs were placed in transport medium and kept at 4ºC.

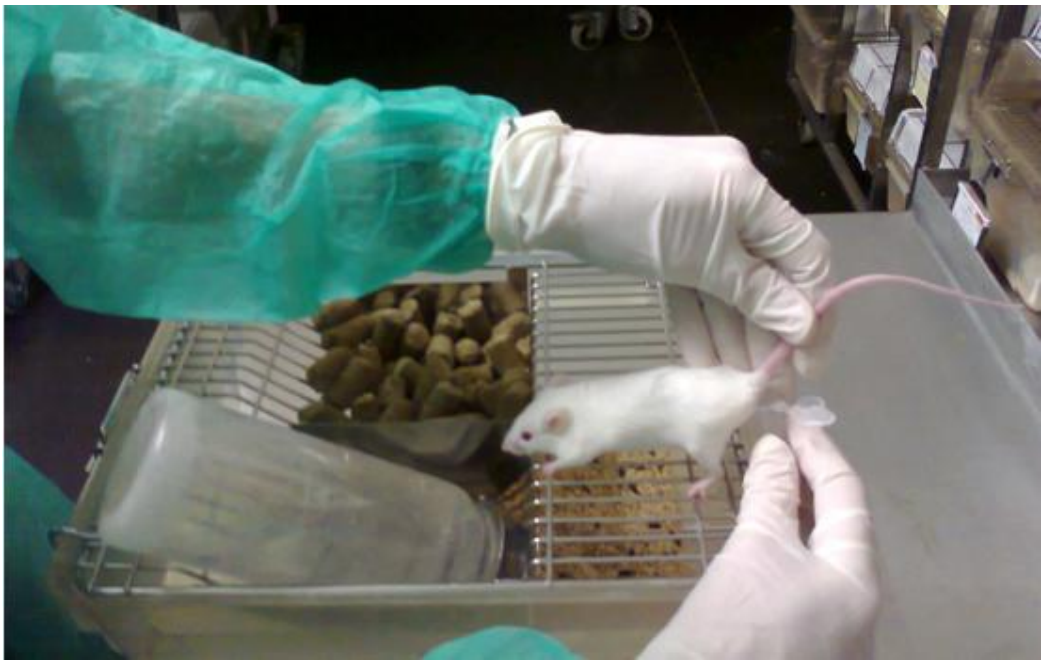


Figure 3: Fecal sample collection.

2.3. DNA isolation and quantification

DNA from individual fecal pellets was isolated using a QIAamp DNA Stool Mini Kit (Qiagen) according to the manufacturer's protocol for isolation of DNA from stool for pathogen detection. DNA concentration and purity were determined spectophotometrically by measuring the absorbance at 260 nm and 280 nm in a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies). One unit of absorbance corresponds to 50 µg/ml for double-stranded DNA.

2.4. Primers

Primers used in this work are described in Table 1. Primer pair Bootz F / Bootz R has been previously described for the specific detection of *Pasteurellaceae* (Bootz *et al.*, 1998) and amplifies a 533 bp fragment of the 16S rRNA gene. The prokaryotic broad-range F27 / R1525 primer pair, targeting also the 16S rRNA gene, was used as PCR positive control and for detection of putative false negative results. All primers were synthesized by IDT – Integrated DNA Technologies.

Table 1: Primers used to amplify regions of the 16S rRNA gene.

Primer	Specificity	Sequence (5' – 3')	Length (bp)	*Tm* (ºC)	%GC	Amplicon size (bp)	Reference
Bootz F	*Pasteurellaceae* 16S rRNA	CAT AAG ATG AGC CCA AG	17	47	47	533	Bootz *et al*., 2001
Bootz R	*Pasteurellaceae* 16S rRNA	GTC AGT ACA TTC CCA AGG	18	50	50		
F27	Eubacterial 16S rRNA	GAG AGT TTG ATC CTG GCT CAG	21	55	52	1500	Lane, 1991
R1525	Eubacterial 16S rRNA	AAG GAG GTG ATC CAG CCG CA	20	61	60		
T7Fwd	pGEM-T Easy Vector	TAA TAC GAC TCA CTA TAG G	19	45	37	533	-
SP6	pGEM-T Easy Vector	ATT TAG GTG ACA CTA TAG	18	42	33		

T_m, Melting temperature calculated by manufacturer; bp, base pairs

2.4.1. Specificity of Bootz primer pair - *in silico* analysis

The specificity of Bootz primer pair was analyzed *in silico* using the EMBL-EBI multiple sequence alignment program ClustalW2 (Chenna *et al.*, 2003). An alignment of 16S rRNA gene sequences available at the NCBI Genbank database from *Pasteurella pneumotropica* (n=26), *Haemophilus parainfluenzae* (n=2), *Haemophilus sp* (n=2), *Actinobacillus muris* (n=2), *Escherichia coli* (n=6), *Proteus mirabilis* (n=2) and *Enterococcus faecalis* (n=1) was performed. The sequences of primers' targets were then searched throughout the aligned sequences. The *Pasteurellaceae* sequences selected for the alignment were submitted to GenBank after the primers description (1998). The Genbank accession numbers and submission dates of the sequences used in the alignment are given in Table 2.

Table 2: Genbank accession numbers of 16S rRNA gene sequences used in the alignment.

Accession	Organism	Strain	Submission date
M75083	*Pasteurella pneumotropica* Jawetz	NCTC 8141	24-01-2000
AF224296	*Pasteurella pneumotropica* Jawetz	NCTC 10827	13-06-2000

AF012090	*Pasteurella pneumotropica* Heyl	CNP 160	28-11-2001
AY362924	*Pasteurella pneumotropica* Jawetz	NCTC 8141	28-07-2004
DQ875933	*Pasteurella pneumotropica* Heyl	Q480011-V1	30-08-2006
FJ685629	*Pasteurella pneumotropica* Heyl	T08711-V2	01-02-2010
FJ685626	*Pasteurella pneumotropica* Jawetz	J426011	01-02-2010
FJ685623	*Pasteurella pneumotropica* Heyl	ATCC 12555	01-02-2010
GU809188	*Pasteurella pneumotropica* Jawetz	CR3	08-04-2011
GU809187	*Pasteurella pneumotropica* Jawetz	CR51	08-04-2011
GU809186	*Pasteurella pneumotropica* Jawetz	CR53	08-04-2011
GU809185	*Pasteurella pneumotropica* Jawetz	CR19	08-04-2011
GU809184	*Pasteurella pneumotropica* Jawetz	CR54	08-04-2011
GU809183	*Pasteurella pneumotropica* Jawetz	CR17	08-04-2011
GU809182	*Pasteurella pneumotropica* Jawetz	CR13	08-04-2011
GU809181	*Pasteurella pneumotropica* Heyl	CR28	08-04-2011
GU809180	*Pasteurella pneumotropica* Heyl	CR26	08-04-2011
GU809179	*Pasteurella pneumotropica* Heyl	CR24	08-04-2011
GU809178	*Pasteurella pneumotropica* Heyl	CR10	08-04-2011
GU809177	*Pasteurella pneumotropica* Heyl	CR5	08-04-2011
GU809176	*Pasteurella pneumotropica* Heyl	CR16	08-04-2011
GU809175	*Pasteurella pneumotropica* Heyl	CR32	08-04-2011
GU809174	*Pasteurella pneumotropica* Heyl	CR1	08-04-2011
GU809173	*Pasteurella pneumotropica* Heyl	CR30	08-04-2011
GU809172	*Pasteurella pneumotropica* Heyl	CR18	08-04-2011
NR_042887	*Pasteurella pneumotropica* Jawetz	NCTC 8141	10-08-2011
FJ685628	*Haemophilus parainfluenzae*	B160041	01-02-2010
FJ685627	*Haemophilus parainfluenzae*	I112013	01-02-2010
FJ685625	*Haemophilus sp*	HK447	01-02-2010
FJ685624	*Haemophilus sp*	HK445	01-02-2010
AY362894	*Actinobacillus muris*	NCTC12432	20-07-2004
NR_042870	*Actinobacillus muris*	NCTC12432	10-08-2011
J01859	*Escherichia coli*	-	11-08-1995
Z83204	*Escherichia coli*	-	01-03-1997
X80732	*Escherichia coli*	MC4100	29-03-1996
X80724	*Escherichia coli*	ATCC 25922	29-03-1996
X80725	*Escherichia coli*	ATCC 11775T	29-03-1996
X80731	*Escherichia coli*	pk3	29-03-1996
AJ301682	*Proteus mirabilis*	CIP103181T	06-06-2003
EU643833	*Proteus mirabilis*	Hu	07-05-2008
Y18293	*Enterococcus faecalis*	-	22-07-1999

2.5. Polymerase Chain Reaction

DNA amplification by PCR was performed in a 25 µL reaction volume. Each reaction contained 1x *Taq* Buffer with KCl (Fermentas), 2 mM $MgCl_2$ (Fermentas), 0.2 pmol/µL of each primer, 0.2 mM of each dNTP (Fermentas), 1U *Taq* DNA polymerase recombinant (Fermentas) and 80 ng of template DNA. Fecal DNA samples from 2 animals housed in a conventional room, and derived from a colony known to be infected with *Pasteurellaceae*, were used as positive controls. Fecal DNA samples from 2 animals purchased from a commercial *Pasteurellaceae*-free colony (Charles River Laboratories, Spain) were used as negative controls. PCR without template DNA was performed as negative PCR control. All samples were tested with Bootz F / Bootz R and F27 / F 1523 primer pairs. PCR amplifications were carried out in a TProfessional Basic Gradient 96 thermocycler (Biometra), in triplicates, under the conditions given in Table 3.

Table 3: Reaction conditions used for PCR amplifications.

Step	Primer pair	
	Bootz F / Bootz R	F27 / R1523
Denaturation	95ºC – 4’	95ºC – 4’
Amplification cycles	40 cycles	30 cycles
Denaturation	94ºC – 1’	95ºC – 1’
Annealing	55ºC – 1’	50ºC – 1’
Elongation	72ºC – 1’	72ºC – 1’30’’
Primer Elongation	72ºC – 4’	72ºC – 4’

2.5.1. Detection of PCR products

PCR products were detected by electrophoresis on a 1.5 % (w/v) agarose (Bioron) gel in 1x TAE buffer [40mM Tris-base pH8.0 (MP Biomedicals Europe), 20 mM acetic acid glacial (Merck), 1 mM EDTA pH8.0 (Prolabo)] containing 0.5 µg/mL ethidium bromide (Sigma-Aldrich). PCR products were visualized by UV transillumination using a GelDoc XR+ System (Bio-Rad). GeneRuler 1 kb DNA Ladder 250 – 10000 bp molecular weight marker (Fermentas) was included on each gel alongside the samples.

2.6. Cloning and sequencing of PCR products

PCR products from different animals were excised from the agarose gel and subsequently purified using illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare) according to the manufacturer's protocol. The purified PCR fragments were ligated in a pGEM-T Easy Vector (Promega) according to the manufacturer's instructions and the resultant constructs were used to transform *E. coli* DH5α competent cells prepared according to the protocol previously described by (Hanahan, 1985). To confirm successful cloning, white colonies were checked by colony PCR using Bootz primer pair as previously described, and 5 μL of a 50 μL colony suspension as template DNA. For each ligation, 3 colonies containing the PCR derived insert were selected and plasmid DNA was isolated using GenElute Plasmid Miniprep Kit (Sima-Aldrich) according to the manufacturer's instructions. To confirm the presence of the insert in the plasmid, a restriction pattern analysis with *Eco*RI (Fermentas) was performed. All assays were performed in duplicates. Sequencing of the inserts was performed by StabVida (Lisbon, Portugal) using the specific pGEM-T Easy Vector primers T7Fwd and SP6 (Table 1). Each nucleotide was sequenced a minimum of 3 times in each strand.

2.6.1. Sequencing analysis and multiple alignment

The sequences were assembled with Vector NTI software (Invitrogen). The homology search of each sequence was performed with the BLASTn tool (Altschul *et al*., 1990) provided by the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Further, the assembled sequences were aligned with 16S rRNA gene sequences of the reference strains *P. pneumotropica* Jawetz NCTC 8141 (AY362924) and *P. pneumotropica* Heyl ATCC 12555 (FJ685623) using the EMBL-EBI multiple sequence alignment program ClustalW2 (Chenna *et al.*, 2003). Multiple alignment was edited with GeneDoc software (Nicholas *et al*., 1997).

2.7. Comparison of the fecal PCR with standard culture methods

The animals used to develop the fecal PCR for *Pasteurellaceae* detection were also analysed by culture techniques in two external certified laboratories. Oral swabs from 10

animals were sent to the Charles River Laboratories (Lyon, France) for *Pasteurella pneumotropica* screening and 10 live animals were sent to QM Diagnostics (Nijmegen, The Netherlands) for *Pasteurellaceae* screening.

2.8. Prevalence of *Pasteurellaceae* in SOPF mice

Prevalence of *Pasteurellacea*e among the SOPF mice of the IBMC's Animal House was assessed using the newly developed fecal PCR assay. A cross sectional study was performed with approximately 10% of the SOPF mice population of the IBMC's Animal House. A total of 360 SOPF animals were analyzed during a one-year period (January to December 2010). All assays were performed as described in 2.5. Each PCR run included one positive control sample, one negative control sample and a no-DNA template control. Statistical analysis was performed with GraphPad Prism 5 Software. The Fisher's exact test was applied to correlate the positive and negative cases with sex, age, breeding status, cage type and immune status of the animals. Statistically significant difference was considered when $p < 0.05$.

3. Results and Discussion

Diagnosis of *Pasteurellaceae* infections is usually based on bacterial culture and subsequent phenotypic characterization. As these procedures are time-consuming and sometimes yield indeterminate results due to the phenotypical diversity of the *Pasteurellaceae* family, molecular based techniques have been reported as alternative diagnostic tools. Several PCR assays have been described for the identification of cultured *Pasteurellaceae* isolated from rodents and also for their detection in clinical samples. However, the sampling methods used for DNA isolation purposes are frequently invasive and require the sacrifice of animals. PCR assays using fecal pellets have been described for several rodent pathogens, including *Helicobacter spp.* (Beckwith *et al.*, 1997), *Clostridium piliforme* (Furukawa *et al.*, 2002) and *Citrobacter rodentium* (McKeel *et al*., 2002). In this study we developed a simple, specific and non-invasive fecal PCR assay to detect *Pasteurellaceae* in laboratory mice using previously described specific primers. In that report, PCR was performed with DNA isolated either from cultured *Pasteurellaceae* from nasopharyngeal swabs or DNA directly isolated from pharynx, trachea and lung specimens, requiring the animals sacrifice (Bootz *et al*., 1998). We also compared our fecal PCR assay with standard diagnostic procedures such as bacterial isolation and identification. Furthermore we discussed the impact of this non-invasive technique in assessing the prevalence of *Pasteurellaceae* in laboratory rodents.

3.1. The feces as a source of bacterial DNA

Fresh fecal pellets were reliably obtained from individual mice and DNA was isolated with a QIamp DNA Stool Mini Kit (Qiagen). This commercial kit includes InhibitEX tablets for the adsorption of impurities typically present in stool samples that can degrade DNA and inhibit downstream enzymatic reactions. Such impurities can include bilirubin, bile salts (Beckwith *et al*., 1997) or complex polysaccharides possibly originating from vegetable materials in the diet (Monteiro *et al*., 1997).

DNA isolation from 362 fecal pellets belonging to different mice yielded an average of 25.74±1.34 ng/µL per fecal pellet (range 0.32 – 184.8 ng/µL). Purity of the DNA preparation, as indicated by the OD260/OD280, had a mean value of 2.074±0.11 (range 0.46 – 37.65) (Figure 4).

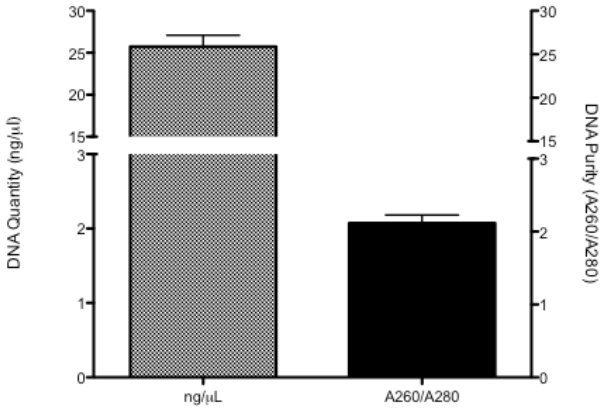


Figure 4: Mean values and standard error of fecal DNA concentration and purity from total mice.

Regarding DNA purity, for 24 samples the OD260/OD280 had values below 1.7 and above 2.1. However, for the remaining 338 samples the OD260/OD280 showed a mean value of 1.84. As such, the obtained results indicate that fecal pellets can be used as a good source of DNA in mice.

3.2. Specificity of Bootz primer pair - *in silico* analysis

The primer pair used for the specific detection of *Pasteurellaceae* was described in 1998 and was selected on the basis of 16S rRNA gene sequences of various rodent isolates representing different phenotypic groups of *Pasteurellaceae* together with all sequences from NCBI GenBank and EMBL-EBI databases available at that time (Bootz *et al.*, 1998). However, the number of entries in the Genbank database increased since then. As such, the primers specificity to target *Pasteurellaceae* was assessed *in silico* using the 16S rRNA gene sequences of *Pasteurellaceae* submitted since 1998 to date (see Table 2). *In silico* analysis was performed by aligning 16S rRNA gene sequences from *P. pneumotropica, H. parainfluenzae, Haemophilus sp, A. muris, E. coli, P. mirabilis* and *E. faecalis,* and subsequently searching for the sequences of primers' targets throughout the aligned sequences. *H. parainfluenzae, Haemophilus sp.* and *A. muris* are members of the *Pasteurellaceae* family and have been previously isolated from laboratory rodents (Csukas, 1975; Bisgaard, 1986; Boot *et al.*, 2005). *E. coli, P. mirabilis* and *E. faecalis* were included for their potential to interfere with the assay as they are commonly found in normal rodent's feces (Beckwith *et al.,* 1997). Observed nucleotide mismatches are summarized in Table 4.

Table 4: Bootz primers mismatches with target sequences of different species. Dots indicate base identity with the primers sequence.

Sequences of primers' target (5'- 3')		Species
Bootz F (positions 215 – 231)*	Bootz R (positions 730-747)*	
CATAAGATGAGCCCAAG	CCTTGGGAATGTACTGAC	
.....	.....	*Pasteurella pneumotropica*
....G.....	.....	*Haemophilus parainfluenzae*
.G.G.....	.....	*Haemophilus sp*
.....	.....	*Actinobacillus muris*
...CG....T.....GA	..C...ACGAAG.....	*Escherichia coli*
T..CG.....A....TA	..C...AC.AAG.....	*Proteus mirabilis*
TGATG....GA...GC.	.TC...TCTGTA.....	*Enterococcus faecalis*

*Base positions are given according to *P.pneumotropica* NCTC 8141 numbering (Genbank accession no. M75083).

DNA polymerases catalyze the addition of nucleotides to the primer 3'-OH, as specified by complementarity to the template DNA. Mismatches between primers and targeted DNA can affect duplex stability, which might then hamper the ability of a system to amplify the template DNA. The effects of mismatches depend on numerous factors, such as oligonucleotide length and the nature and position of the mismatches. Several studies have investigated the effects of primer-template mismatches at the 3' end of the primer sequence, and it has been demonstrated that PCR was prevented by a single mismatched base at the 3' end. In contrast, mismatches at the 5' end and internal mismatches can be tolerated (Kwok *et al*., 1990). As shown in Table 4, the sequences of Bootz primers' target are 100% identical to the 16S rRNA gene sequences of *P. pneumotropica* and *A. muris* at positions 215-231 for the forward primer and 730-747 for the reverse primer. Furthermore, the forward primer differs only by 1 base from the sequence of *H. parainfluenzae* and by 2 bases from that of *Haemophilus sp* near the 5' end. The reverse primer has no mismatches with the sequences both species. However, the number of nucleotide mismatches with organisms other than *Pasteurellaceae* is significantly higher. Particularly in the forward primer, the mismatches are located at the 3' end of the sequences. Therefore, the PCR reaction is not likely to occur.

The *in silico* analysis confirms that this primer pair specifically targets the 16S rRNA region of members of the *Pasteurellaceae* family, as previously described (Bootz *et al*., 1998). Due to its specificity, this primer pair meets the specifications to be used in PCR assays for the detection of *Pasteurellaceae*. Therefore it was selected to develop our fecal PCR assay.

3.3. Evaluation of the fecal PCR for *Pasteurellaceae* detection

To develop our fecal PCR assay, a total of 20 mice were examined (mice numbered from 1 to 20): 2 mice (numbers 1 and 2) kept in a non-barrier conventional room and 18 mice (numbers 3 to 20) with SOPF health status, kept in barrier-rooms. Samples from animals 1 and 2 were used as positive controls because they derived from colonies known to be infected with *P. pneumotropica* and presented clinical signs of conjunctivitis. From the 18 SOPF mice, 2 (numbers 4 and 5) were purchased from a commercial *Pasteurellaceae*-free colony (Charles River Laboratories, Spain) and fecal samples were collected after one week of quarantine at the IBMC's Animal House. These samples were used as negative controls.

3.3.1. Assay sensitivity

To estimate the sensitivity of the fecal PCR, different quantities of template DNA ranging from 5 to 100 ng were used in a series of reactions with Bootz primer pair. In this assay we used one of the positive control samples (mouse number 2) as template DNA. Results are shown in Figure 5.

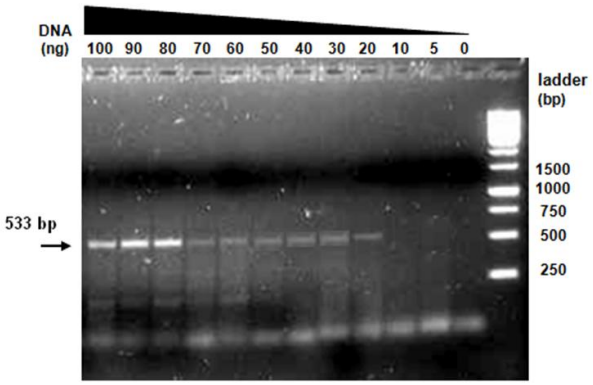


Figure 5: PCR amplification using different amounts of template DNA from mouse no.2. Bootz primers amplified a 533 bp fragment.

According to this assay, the lower limit of detection of our test was 20 ng of template DNA. Based on the obtained results we estimate that the PCR assay should be performed with 80 to 100 ng of template DNA in each reaction, where a stronger signal is observed.

3.3.2. Detection of *Pasteurellaceae* in fecal samples

After establishing the optimal conditions for the PCR assay, all 20 samples were analyzed with Bootz primer pair using 80 ng of template DNA. The results obtained are shown in Figure 6A. The expected 533 bp DNA fragment was amplified from 12 fecal DNA samples: the positive control samples (mice 1 and 2) and samples from 10 other animals (mice 3, 6, 8, 9, 11, 13, 14, 15, 16 and 17). On the contrary, this PCR assay was not able to amplify any fragment from the negative control samples (mice 4 and 5), as expected, and from the remaining 6 samples (mice 7, 10, 12, 18, 19 and 20).

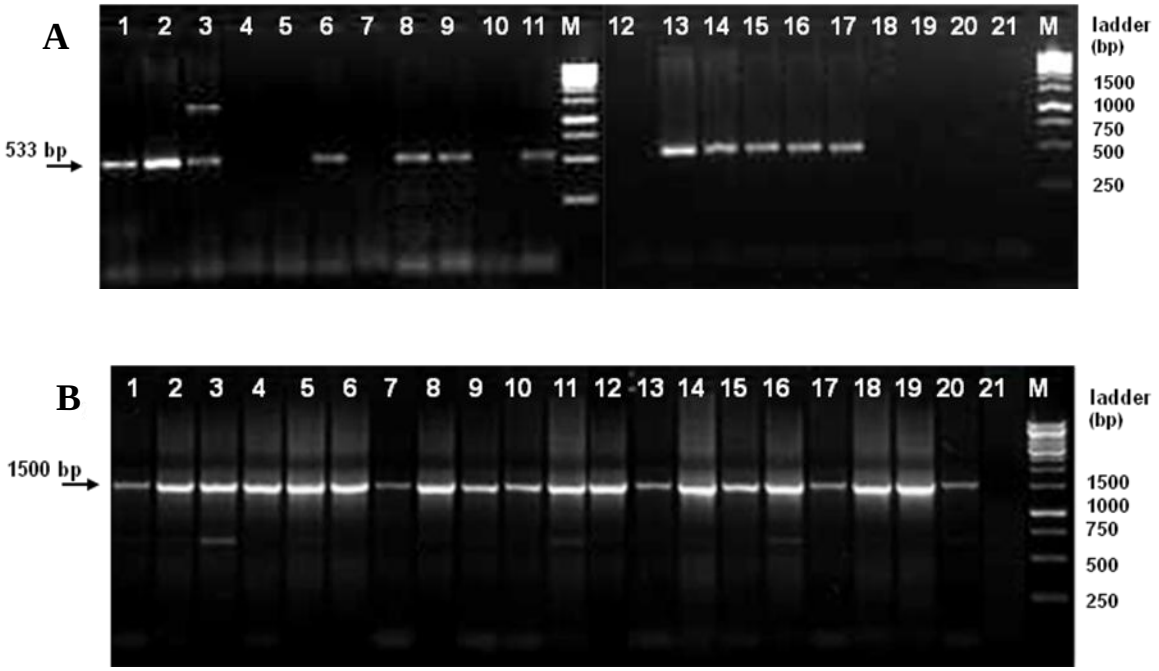


Figure 6: PCR amplification of fecal DNA samples from 20 different mice. Lanes: 1 – 20, animals 1 to 20 respectively; 21, no template DNA; M, 1kb DNA ladder. **A:** PCR amplification with Bootz primer pair yielding a 533 bp fragment. **B:** PCR amplification with prokaryotic broad-range primer pair yielding a 1500 bp fragment.

Fecal PCR analytic methods can be used as a non-invasive means of rapidly screening large numbers of mice. However, one of the main concerns of all fecal tests is the potential of obtaining false negative results due to the presence of PCR inhibitors in feces. To exclude putative false negative results, PCR reactions with prokaryotic broad-range F27 / R1525 primer pair were performed for all samples. In Figure 6B it is shown that the expected 1500 bp fragment was amplified from all the tested samples, including those which yielded no

amplicon when PCR was performed with Bootz primer pair. The obtained results show that fecal DNA samples belonging to mice 4, 5, 7, 10, 12, 18, 19 and 20 are in fact *Pasteurellaceae* free samples, and confirm that the isolated fecal DNA is suitable to monitor bacteria in mice stool samples.

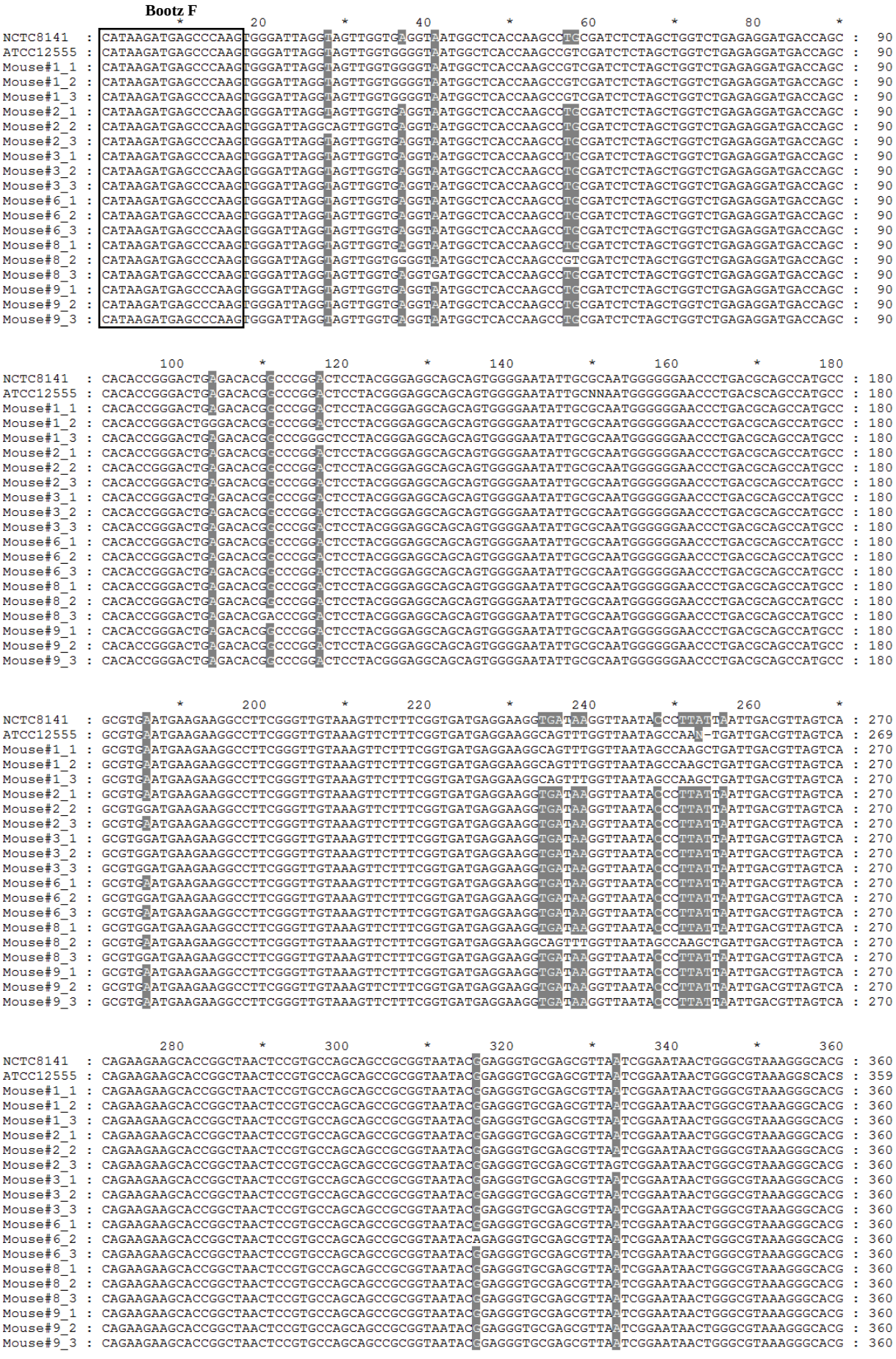
According to Bootz *et al*. (1998), *Haemophilus influenzaemurium* strains yielded an additional band approximately 1200 bp long. A band with this approximate size was amplified from the sample of animal number 3 (Figure 6A).

3.4. Sequencing analysis and multiple alignment

PCR products from randomly chosen individuals yielding the expected 533 bp DNA fragment (animals 1, 2, 3, 6, 8 and 9) were sequenced to confirm the specificity of the PCR assay and to disclose the occurrence of false positive results. In addition to the expected 533 bp amplicon, the amplicon of approximately 1200 bp obtained from animal number 3 IFigure 6) was also sequenced. Sequences from 18 clones of the 533 bp fragment (3 clones per animal) and from 3 clones of the 1200 bp fragment were assembled with Vector NTI software (Invitrogen) and compared with NCBI GenBank entries by using the BLASTn algorithm. Results for the first hit of each sequence are shown in Table 5. Further, the assembled sequences were aligned with 16S rRNA gene sequences of *P. pneumotropica* Jawetz and Heyl reference strains using the EMBL-EBI ClustalW2 software (Figure 7).

Table 5: NCBI GenBank sequences producing significant alignments with the sequenced 533 bp and 1200 bp fragments.

Mouse	Fragment length (bp)	Accession	Organism	Query coverage	E value	Maximum identity
1	533	GU809177	*P. pneumotropica* Heyl strain CR5	100%	0.0	99%
2	533	GU809188	*P. pneumotropica* Jawetz strain CR3	100%	0.0	99%
3	533	GU809188	*P. pneumotropica* Jawetz strain CR3	100%	0.0	99%
	1369	CP000612	*Desulfotomaculum reducens* MI-1	10%	6e-13	73%
6	533	GU809188	*P. pneumotropica* Jawetz strain CR3	100%	0.0	99%
8	533	GU809188	*P. pneumotropica* Jawetz strain CR3	100%	0.0	99%
	533	GU809177	*P. pneumotropica* Heyl strain CR5	100%	0.0	99%
9	533	GU809188	*P. pneumotropica* Jawetz strain CR3	100%	0.0	99%


Bootz F

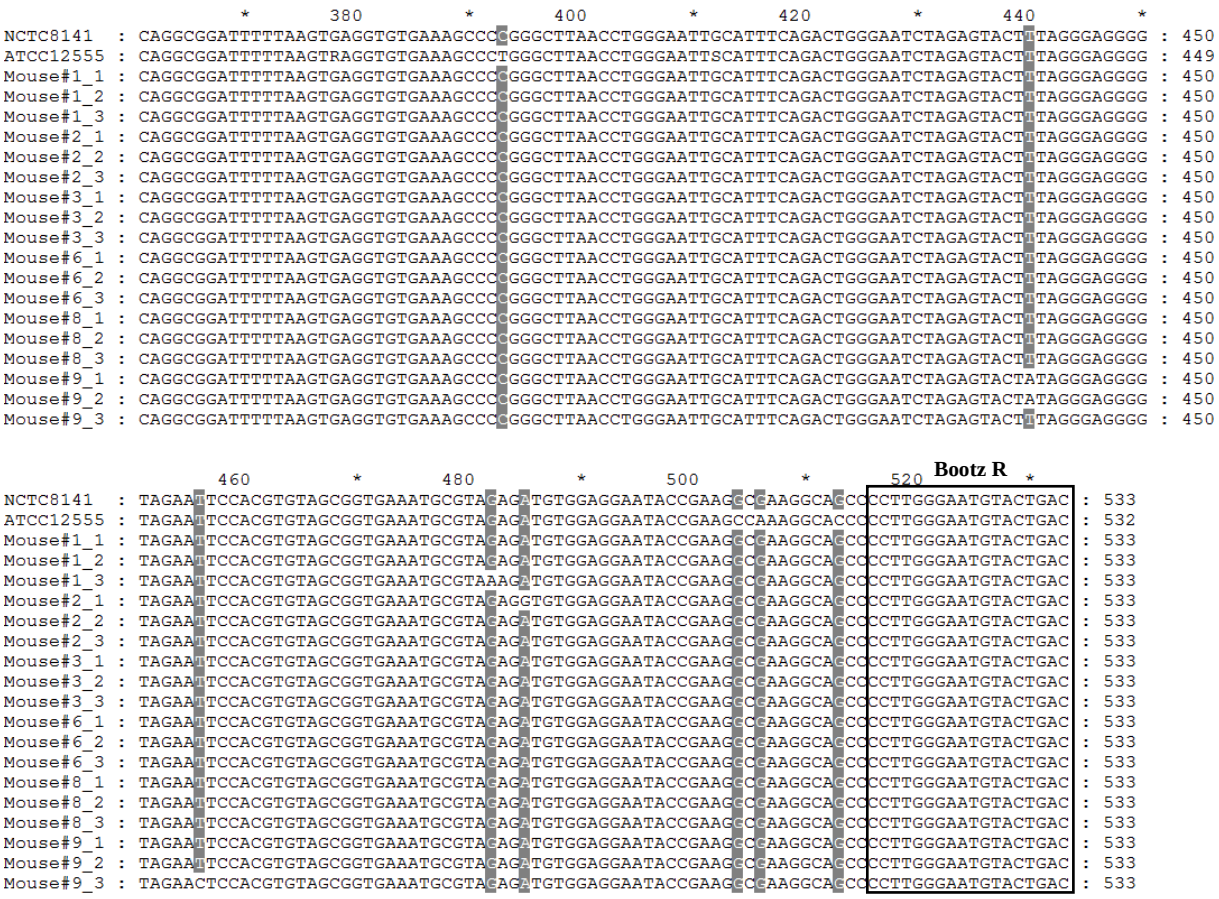


Figure 7: Multiple alignment of the 533 bp sequenced fragments and reference strains *P. pneumotropica* Jawetz NCTC 8141 (AY362924) and *P. pneumotropica* Heyl ATCC 12555 (FJ685623). Wobble bases: R=A+G, S=C+T, N=A+G+C+T. The sequences of primers Bootz F and Bootz R are boxed.

Results from the BLASTn analysis (Table 5) show a 99% identity in the 533 bp overlap between the sequences of the fragments amplified from mice feces and GenBank sequences of the 16S rRNA gene of *P. pneumotropica*. Furthermore, the Expected value (E) is 0.0 for all sequences. The Expect value is a parameter that describes the number of hits one can expect to see by chance when searching a database of a particular size. The lower the E-value, or the closer it is to zero, the more significant the match is. Thus, BLASTn analysis of the sequenced 533 bp fragments confirms the specificity of the PCR assay. However, the other fragment obtained from animal number 3 was in fact 1369 bp long and revealed no identity with members of the *Pasteurellaceae* family.

Multiple alignment of the sequenced 533 bp fragments with 16S rRNA gene sequences of reference strains *P. pneumotropica* Jawetz NCTC 8141 (AY362924) and *P. pneumotropica* Heyl ATCC 12555 (FJ685623) (Figure 7) show 14 single nucleotide polymorphisms (SNPs)

between the sequences of both biotypes. Further, the sequences of the three clones from the fragment amplified from mouse number 1 and of one clone from the fragment amplified from mouse number 8 are highly similar to *P. pneumotropica* Heyl ATCC 12555 (FJ685623). All the other clones show high similarity to *P. pneumotropica* Jawetz NCTC 8141 (AY362924). These results confirm the BLAST analysis of the sequences, suggesting that animals 2, 3, 6 and 9 were infected with *P. pneumotropica* biotype Jawetz whereas animal 1 was infected with *P. pneumotropica* biotype Heyl. Animal 8 could possibly be infected with both biotypes.

3.5. Comparison of the fecal PCR with standard culture methods

To confirm the results obtained with the novel fecal PCR assay, the 20 analyzed animals (see 3.3.2.) were also tested by standard culture methods in two external certified laboratories. Oral swabs from 10 of these animals (numbers 1 to 10) were sent for *P. pneumotropica* screening at the Charles River Laboratories (Lyon, France) and the other 10 live animals (numbers 11 to 20) were sent for *Pasteurellaceae* screening at QM Diagnostics (Nijmegen, The Netherlands). The culture results were compared with those obtained by fecal PCR (Tables 6 and 7). All culture positive samples also tested positive by fecal PCR. However, the PCR assay was able to detect 2 more positive samples (20%) than the cultures performed at Charles River, and 3 more positive samples (30%) than the cultures performed at QM Diagnostics.

Table 6: Comparison of results obtained for *Pasteurellaceae* screening by fecal PCR and culture for *P. pneumotropica* screening performed by the Charles River Laboratories.

Mouse no.	1*	2*	3*	4	5	6*	7	8*	9*	10
Fecal PCR result	+	+	+	-	-	+	-	+	+	-
Culture result	+	+	+	-	-	-	-	-	+	-

*animals yielding the 533bp amplicons that were sequenced

Table 7: Comparison of results obtained for *Pasteurellaceae* screening by fecal PCR and culture for *Pasteurellaceae* screening performed by QM Diagnostics.

Mouse no.	11	12	13	14	15	16	17	18	19	20
Fecal PCR result	+	-	+	+	+	+	+	-	-	-
Culture result	-	-	-	-	+	+	+	-	-	-

It is known that bacterial PCR assays are capable of detecting as few as 3-10 bacteria (Compton and Riley, 2001). As such, these results suggest that our fecal PCR assay can be more sensitive than the bacteriological examinations routinely performed for *Pasteurellaceae* detection in laboratory rodents. Further, it is possible that this fecal PCR detected an early stage of *Pasteurellaceae* infection that was not detected by culture methods. To demonstrate this hypothesis, animals 6, 8, 11, 13 and 14 could have been followed up and tested again latter with both methods.

The fact that this new fecal PCR assay works without prior culture of the organism not only increases sensitivity of detection but also significantly reduces the time required for the assay. For laboratories familiar with the methods used in molecular biology, the PCR assay is easy to perform and does not require expertise in identifying bacterial colonies. If an animal or a colony tests negative, further work is not necessary and a colony can be declared *Pasteurellaceae* free with a high degree of confidence. If an animal tests positive, one may choose to identify the strain, which can be done by traditional methods such as culture and biochemical characterization. A more straightforward option is sequencing of the PCR product generated and comparing it with published sequences of other strains. On the basis of this information, the laboratory animal veterinarian, together with the investigator using the animals, can decide on the best course of action. Issues to be considered are manifestation of clinical disease (especially in immunodeficient and/or genetically modified animals) and the potential of interfering with the research projects involved.

On the basis of these findings, we conclude that our fecal PCR assay would greatly facilitate and improve the screening of *Pasteurellaceae* in laboratory rodents. It is more sensitive than the culture isolation, less laborious and not as time-consuming as the traditional methods, making it the ideal screening method for routine testing. A positive PCR result may then be followed up if necessary.

3.6. Prevalence of *Pasteurellaceae* in SOPF mice

Periodic health screening of rodents used in research is necessary due to the consequences of unwanted infections on animal welfare and research results. Contemporary disease prevalence should be a primary consideration in designing efficient and effective health monitoring schemes. In a recent microbiologic screen of 109.403 mice from institutions in North America and Europe, the prevalence of *P. pneumotropica* was found to be 12.9%

emphasizing the need to monitor this microorganism (Pritchett-Corning *et al.*, 2009). As such, we decided to evaluate the potential of this non-invasive technique in assessing the prevalence of *Pasteurellaceae* in mice from our animal facility.

A cross sectional study was performed with approximately 10% of the SOPF mice population of the IBMC's Animal House. A total of 360 SOPF animals were analyzed during a one-year period (January to December 2010) using the newly developed fecal PCR. All assays were performed as described in 2.5. using 80 ng of template whenever possible. Taking into account the obtained DNA concentrations, 5 samples were analyzed with less than 20 ng of template DNA and 20 samples were analyzed with DNA amounts ranging from 20 to 80 ng. Although 24 samples showed OD260/OD280 values below 1.7 and above 2.1 (see 3.1.), all samples yielded a positive result with the prokaryotic broad-range primers. PCR assays with Bootz primer pair yielded 65 positive results and therefore the prevalence of *Pasteurellaceae* infection was determined to be 18%. Results of prevalence according to sex, age, breeding status, cage type and immune status are given in the following sections.

3.6.1. Sex distribution of *Pasteurellaceae* infection

Table 8: Prevalence of *Pasteurellaceae* infection in SOPF mice according to sex.

Sex	Male	Female	*p value
Number of animals	197	163	
Number of positive results	28	37	0.0399
Prevalence	**14.21%**	**22.70%**	

*Fisher's exact test

Pasteurellaceae are among the most frequently occurring bacterial species found in the female genital tract of laboratory mice and rats (Yamada *et al*., 1983) and have been associated with infection of the reproductive tract in female laboratory rodents (Ward *et al*., 1978; Mikazuki *et al*., 1994). In Table 8 it is shown that the prevalence of *Pasteurellaceae* infection was higher in females than in males and such difference was statistically significant ($p < 0.05$). Based on this observation we further addressed whether *Pasteurellaceae* infected females yielded more DNA than *Pasteurellaceae* infected males but statistical analysis showed no significant difference. However, as the DNA yield refers to total DNA isolated from a fecal pellet, further studies would be needed to address this question.

3.6.2. Age distribution of *Pasteurellaceae* infection

Table 9: Prevalence of *Pasteurellaceae* infection in SOPF mice according to age group.

Age group (months)	≤ 3	> 3	*p value
Number of animals	174	186	
Number of positive results	36	29	0.2200
Prevalence	**20.69%**	**15.59%**	

*Fisher's exact test

According to mice age, prevalence of *Pasteurellaceae* infection was higher in animals with less than 3 months than in older animals (Table 9). However, the observed difference was not statistically significant.

3.6.3. Breeding status and *Pasteurellaceae* infection

Table 10: Prevalence of *Pasteurellaceae* infection in SOPF males and females according to breeding status.

Breeding Status	Breeders			Non-breeders		
Sex	Male	Female	*p value	Male	Female	*p value
Number of animals	121	102		76	61	
Number of positive results	16	28	0.0108	12	9	1.000
Prevalence	**13.22%**	**27.45%**		**15.79%**	**14.75%**	

*Fisher's exact test

The overall difference between breeder and non-breeder animals was not statistically significant ($p = 0.3250$). The results shown in Table 10 indicate that among breeder animals, the prevalence of *Pasteurellaceae* infection was significantly higher in females than in males ($p < 0.05$). These results correlate with those observed in section 3.6.1. However, although the difference was not statistically significant, among non-breeder animals the prevalence of *Pasteurellaceae* infection was higher in males.

3.6.4. Impact of housing conditions in *Pasteurellaceae* prevalence

Table 11: Prevalence of *Pasteurellaceae* infection in SOPF mice according to cage type.

Cage type	Top filtered cage	IVC	*p value
Number of animals	239	121	
Number of positive results	31	34	0.0007
Prevalence	**12.97%**	**28.10%**	

* Fisher's exact test

The impact of housing conditions in *Pasteurellaceae* infection is shown in Table 11. The prevalence of *Pasteurellaceae* was significantly higher in animals housed in Individually Ventilated Cages (IVCs) than in animals housed in top filtered cages ($p < 0.001$). These results are very difficult to explain as IVCs are known to be more effective to prevent the spread of microorganism than top filtered cages (Hasegawa *et al*., 2003). We hypothesize that the observed difference can be associated to a poor sanitation and maintenance of the IVC systems, and that in these conditions *Pasteurellaceae* are able to survive in the HEPA filters or in the IVC connection air conducts. However, to address this question, further studies would need to be performed.

3.6.5. Impact of mice immunological status in *Pasteurellaceae* prevalence

Table 12: Prevalence of *Pasteurellaceae* infection in SOPF mice according to immune status.

Immune status	Immunocompromised	Immunocompetent	*p value
Number of animals	132	228	
Number of positive results	17	48	0.0641
Prevalence	**12.88%**	**21.05%**	

* Fisher's exact test

Surprisingly, immunocompetent animals showed higher prevalence of infection than immunodeficient mice (Table 12). However, statistical analysis showed that this difference was not statistically significant.

4. Concluding remarks

Pasteurella pneumotropica is considered the most pathogenic member of *Pasteurellaceae* in rodents. It is often specifically looked for in routine health monitoring while other members of this family are disregarded. However, other members of the family have been described as causing clinical disease (Bootz *et al*., 1998). Although there is no universal agreement, we believe that other members of the *Pasteurellaceae* may also be of importance. If not principally as agents causing overt disease, they may influence biomedical research by causing subclinical infection. The most promising approach for detecting infection with *Pasteurellaceae* might be PCR. This method was documented to detect *Pasteurella pneumotropica* in culture-negative animals (Bootz *et al*., 1998). Several diagnostic PCR assays for *Pasteurellaceae* screening have been described (Wang *et al*., 1996; Bootz *et al*., 1998; Weigler *et al.*, 1998; Kodjo *et al*., 1999; Hayashimoto *et al*, 2007a) but they all rely on invasive sampling methods that require the sacrifice of animals.

The PCR assay described in this study uses feces as the test sample. Stool samples are easy to obtain without excessive distress, pain or injury to the animals, and our results show that this method can be reliably applied in the detection of *Pasteurellaceae* in mice. Since feces can be collected from live animals, the major advantage of our fecal PCR assay is that it does not require euthanasia of the animals being tested. The ability to test live animals is especially important for monitoring valuable and rare rodents, such as transgenic and knockout mice, and it contributes to the reduction of the number of animals used for health monitoring purposes and to the refinement of animal welfare according to the 3Rs policy. Furthermore, it allows the specific detection of *Pasteurellaceae* directly from the fecal samples, thereby eliminating the need for bacteriology and offering a more sensitive and faster detection method than classical culture techniques. It is also a valuable epidemiological tool to assess the prevalence of *Pasteurellaceae* in mice colonies.

In summary, stool samples are a preferable source for DNA isolation. This non-invasive method reduces discomfort and stress to the laboratory animal and reduces animal loss for *Pasteurellaceae* screening purposes. We conclude that performing PCR with fecal DNA could be a useful technique for identifying animals infected with *Pasteurellaceae*.

5. Future work

The use of diagnostic tools that rely on easily accessible samples from laboratory animals is of great interest in health monitoring programs. Invasive sampling methods constitute a frequent problem in assessing the health status of rodent colonies, with undesirable loss of animals that are important in biomedical research. This study suggest a non-invasive diagnostic tool for *Pasteurellaceae* detection in mice that can be further investigated in the assessment of its potential applicability for screening infected rats or other laboratory rodents.

This fecal the PCR assay could be optimized to a one-step duplex reaction including both described primers pairs in order to reduce the time required for the assay and also the costs involved.

Further, this assay could be used to assess the shedding cycle of *Pasteurellaceae* in rodents feces by collecting and analyzing samples during specified periods.

Finally, conventional mice could also be tested in order to conduct a comparative epidemiological study between the prevalence of *Pasteurellaceae* infection in the SOPF and conventional areas of the IBMC's Animal House. Questions regarding the impact of housing conditions and immunological status in the prevalence of *Pasteurellaceae* among SOPF mice could be further investigated.

6. References

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J. 1990. Basic local alignment search tool. *Journal of Molecular Biology* **215**(3):403-410.

Artwohl, J.E., Flynn, J.C., Bunte, R.M., Angen, O., Herold, K.C. 2000. Outbreak of *Pasteurella pneumotropica* in a closed colony of STOCK-Cd28(tm1Mak) mice. *Contemporary Topics in Laboratory Animals Science* **39**(1):39-41.

Baker, D.G. 1998. Natural pathogens of laboratory mice, rats and rabbits and their effects on research. *Clinical Microbiology Reviews* **11**(2):231-266.

Beckwith, C.S., Franklin, C.L., Hook, R.R. Jr., Besch-Williford, C.L., Riley, L.K. 1997. Fecal PCR assay for diagnosis of *Helicobacter* infection in laboratory rodents. *Journal of Clinical Microbiology* **35**(6):1620-1623.

Bisgaard, M. 1986. *Actinobacillus muris* sp. nov. isolated from mice. *Acta Pathologica et Microbiologica Scandinavica – Section B: Microbiology & Immunology* **94**(1):9-17.

Boot, R., Thuis, H.C., Teppema, J.S. 1993. Hemagglutination by *Pasteurellaceae* isolated from rodents. *Zentralblatt fur Bakteriologie* **279**(2):259-273.

Boot, R., Thuis, H.C., Veenema, J.L., Bakker, R.H.G., Walvoort, H.C. 1994. Colonization and antibody response in mice and rats experimentally infected with *Pasteurellaceae* from different rodent species. *Laboratory Animals* **28**(2):130-137.

Boot, R., Thuis, H.C., Bakker, R.H.G., Veenema, J.L. 1995a. An enzyme-linked immunosorbent assay (ELISA) for monitoring antibodies to SP group *Pasteurellaceae* in guineapigs. *Laboratory Animals* **29**(1):59-65.

Boot, R., Thuis, H.C., Veenema, J.L., Bakker, R.H.G. 1995b. An enzyme-linked immunosorbent assay (ELISA) for monitoring rodent colonies for *Pasteurella pneumotropica* antibodies. *Laboratory Animals* **29**(3):307-313.

Boot, R., Bisgaard, M. 1995c. Reclassification of 30 *Pasteurellaceae* strains isolated from rodents. *Laboratory Animals* **29**(3):314-319.

Boot, R., van den Berg, L., Van Lith, H.A., Veenema, J.L. 2005. Rat strains differ in antibody response to natural *Haemophilus* species infection. *Laboratory Animals* **39**(4):413-420.

Boot, R., van den Berg, L. 2006. Evaluation of antigen panels for ELISA monitoring of mouse colonies for antibodies to *Pasteurellaceae*. *Laboratory Animals* **40**(2):194-199.

Boot, R., Vlemminx, M.J., Reubsaet, F.A. 2009. Comparison of polymerase chain reaction primer sets for amplification of rodent *Pasteurellaceae. Laboratory Animals* **43**(4):371-375.

Bootz, F., Kirschnek, S., Nicklas, W., Wyss, S.K., Homberger, F.R. 1998. Detection of *Pasteurellaceae* in rodents by polymerase chain reaction analysis. *Laboratory Animal Science* **48**(5):542-546.

Brennan, P.C., Fritz, T.E., Flynn, R.J. 1969. Role of *Pasteurella pneumotropica* and *Mycoplasma pulmonis* in murine pneumonia. *Journal of Bacteriology* **97**(1):337-349.

Campos, A., Taylor, J.H., Campbell, M. 2000. Hamster bite peritonitis: *Pasteurella pneumotropica* peritonitis in a dialysis patient. *Pediatric Nephrology* **15**(1-2):31-32.

Carthew, P., Gannon, J. 1981. Secondary infection of rat lungs with *Pasteurella pneumotropica* after Kilham virus infection. *Laboratory Animals* **15**(3):219-221.

Carthew, P., Aldred, P. 1988. Embryonic death in pregnant rats owing to intercurrent infection with Sendai virus and *Pasteurella pneumotropica*. *Laboratory Animals* **22**(1):92-97.

Chapes, S.K., Mosier, D.A., Wright, A.D., Hart, M.L. 2001. MHCII, *Tlr4* and *Nramp1* genes control host pulmonary resistance against the opportunistic bacterium *Pasteurella pneumotropica*. *Journal of Leukocyte Biology* **69**(3):381-386.

Chenna, R., Sugawara, H., Koike, T., Lopez, R., Gibson, T.J., Higgins, D.G., Thompson, J.D. 2003. Multiple sequence alignment with the Clustal series of programs. *Nucleic Acids Research* **31**(13):3497-500.

Christensen, H., Kuhnert, P., Olsen, J.E., Bisgaard, M. 2004. Comparative phylogenies of the housekeeping genes *atpD*, *infB* and *rpoB* and 16S rRNA gene within the *Pasteurellaceae. International Journal of Systematic and Evolutionary Microbiology* **54**(Pt 5):1601-1609.

Christensen, H., Kuhnert, P., Busse, H.J., Frederiksen, W.C., Bisgaard, M. 2007. Proposed minimal standards for the description of genera, species and subspecies of the *Pasteurellaceae*. *International Journal of Systematic and Evolutionary Microbiology* **57**(Pt 1):166-178.

Christensen, H. 2009. "Taxa covered by the ICSP Subcommittee on the Taxonomy of *Pasteurellaceae*." Available: http://www.the-icsp.org/taxa/Pasteurellaceaelist.htm [date visited: 12/08/11].

Compton, S.R., Riley, L.K. 2001. Detection of infectious agents in laboratory rodents: traditional and molecular techniques. *Comparative Medicine* **51**(2):113-119.

Csukas, Z. 1976. Reisolation and characterization of *Haemophilus influenzaemurium*. *Acta Microbiologica Academiae Scientiarum Hungarica* **23**(1):89-96.

Dammann, P., Hilken, G., Hueber, B., Kohl, W., Bappert, M.P., Mahler, M. 2011. Infectious microorganisms in mice (*Mus musculus*) purchased from commercial pet shops in Germany. *Laboratory Animals* **45**(4):271-275.

Dewhirst, F.E., Paster, B.J., Olsen, I., Fraser, G.J. 1992. Phylogeny of 54 representative strains of species in the family *Pasteurellaceae* as determined by comparison of 16S rRNA sequences. *Journal of Bacteriology* **174**(6):2002-2013.

Dewhirst, F.E., Paster, B.J., Olsen, I., Fraser, G.J. 1993. Phylogeny of the *Pasteurellaceae* as determined by comparison of 16S ribosomal ribonucleic acid sequences. *Zentralblatt fur Bakteriologie* **279**(1):35-44.

Dole, V.S., Banu, L.A., Fister, R.D., Nicklas, W., Henderson, K.S. 2010. Assessment of *rpoB* and *16S rRNA* genes as targets for PCR-based identification of *Pasteurella pneumotr*opica. *Comparative Medicine* **60**(6):427-435.

Dousse, F., Thomann, A., Brodard, I., Korczak, B.M., Schlatter, Y., Kuhnert, P.,Miserez, R., Frey, J. 2008. Routine phenotypic identification of bacterial species of the family *Pasteurellaceae* isolated from animals. *Journal of Veterinary Diagnostic Investigation* **20**(6):716-724. Erratum in: *Journal of Veterinary Diagnostic Investigation*. 2009. **21**(2):288.

Euzéby, J.P. 2011. "List of Prokaryotic Names Standing in Nomenclature". Available: http://www.bacterio.cict.fr/classifgenerafamilies.html#Pasteurellaceae [date visited: 12/08/11].

Frebourg, N.B., Berthelot, G., Hocq, R., Chibani, A., Lemeland, J.F. 2002. Septicemia due to *Pasteurella pneumotropica*: 16S rRNA sequencing for diagnosis confirmation. *Journal of Clinical Microbiology* **40**(2):687-689.

Frey, J., Kuhnert, P. 2002. RTX toxins in *Pasteurellaceae. International Journal of Medical Microbiology* **292**(3-4):149-158.

Frey, J. 2011. The role of RTX toxins in host specificity of animal pathogenic *Pasteurellaceae*. *Veterinary Microbiology* **153**(1-2):51-58.

Furukawa, T., Furumoto, K., Fujieda, M., Okada, E. 2002. Detection by PCR of the Tyzzer's Disease organism (*Clostridium piliforme*) in feces. *Experimental Animals* **51**(5):513-516.

Gautier, A.L., Dubois, D., Escande, F., Avril, J.L., Trieu-Cuot, P., Gaillot, O. 2005. Rapid and accurate identification of human isolates of *Pasteurella* and related species by sequencing the *sodA* gene. *Journal of Clinical Microbiology* **43**(5):2307-2314.

Gillard, T., Duval, V., Jobart, R., Brasme, L., David, C., de Champs, C., Begin, M., Dehoux, E. 2009. Dog bite wound infection by *Pasteurella dagmatis* misidentified as *Pasteurella pneumotropica* by automated system Vitek 2. *Diagnostic Microbiology and Infectious Disease* **65**(3):347-348.

Gillard, T., Martin, M., Duval, V., Brasme, L., David, C., Vernet-Garnier, V., Lebargy, F., de Champs, C. 2010. Respiratory tract colonization by *Pasteurella pneumotropica* in a patient with an alpha 1-antitrypsin deficiency unexpectedly well identified by automated system Vitek 2. *Diagnostic Microbiology and Infectious Disease* **68**(2):190-192.

Goelz, M.F., Thigpen, J.E., Mahler, J., Rogers, W.P., Locklear, J., Weigler, B.J., Forsythe D.B. 1996. Efficacy of various therapeutic regimens in eliminating *Pasteurella pneumotropica* from the mouse. *Laboratory Animal Science* **46**(3):280-285.

Hanahan, D. 1985. Techniques for Transformation of *Escherichia coli*. In: DNA Cloning – A Practical Approach (Ed. D.M. Glover), IRL Press, Oxford. pp. 109-135.

Hart, M.L., Mosier, D.A., Chapes, S.K. 2003. Toll-like receptor 4-positive macrophages protect mice from *Pasteurella pneumotropica*-induced pneumonia. *Infection and Immunity* **71**(2):663-670.

Hasegawa, M., Kagiyama, S., Tajima, M., Yoshida, K., Minami, Y., Kurosawa, T. 2003. Evaluation of a forced-air-ventilated micro-isolation system for protection of mice against *Pasteurella pneumotropica*. *Experimental Animals* **52**(2):145-151.

Hayashimoto, N., Aiba, T., Itoh, K., Kato, M., Kawamoto, E., Kiyokawa, S., Morichika, Y., Muraguchi, T., Narita, T., Okajima, Y., Takakura, A., Itoh, T. 2005a. Identification procedure for *Pasteurella pneumotropica* in microbiologic monitoring of laboratory animals. *Experimental Animals* **54**(2):123-129.

Hayashimoto, N., Takakura, A., Itoh, T. 2005b. Genetic Diversity on 16S rDNA Sequence and Phylogenic Tree Analysis in *Pasteurella pneumotropica* strains isolated from laboratory animals. *Current Microbiology* **51**(4):239-243.

Hayashimoto, N., Ueno, M., Takakura, A., Itoh, T. 2006. Phylogenetic analysis of isolates of *Pasteurella pneumotropica* from laboratory animals based on the *gyrB* gene sequence. *Experimental Animals* **55**(5):487-490.

Hayashimoto, N., Ueno, M., Takakura, A., Itoh, T. 2007a. A specific polymerase chain reaction based on the *gyrB* gene sequence and subsequent restriction fragment length polymorphism analysis of *Pasteurella pneumotropica* isolates from laboratory mice. *Journal of the American Association for Laboratory Animal Science* **46**(2):54-58. Erratum in: *Journal of the American Association for Laboratory Animal Science*. 2009. **48**(2):137.

Hayashimoto, N., Ueno, M., Takakura, A., Itoh, T. 2007b. Biochemical characterization and phylogenetic analysis based on 16S rRNA sequences for V-factor dependent members of *Pasteurellaceae* derived from laboratory rats. *Current Microbiology* **54**(6):419-423.

Hayashimoto, N., Yasuda, M., Ueno, M., Goto, K., Takakura, A. 2008. Experimental infection studies of *Pasteurella pneumotropica* and V-factor dependent *Pasteurellaceae* for F344-rnu rats. *Experimental Animals* **57**(1):57-63.

Heyl, J.G. 1963. A study of *Pasteurella* strains from animal sources. *Antonie Van Leeuwenhoek* **29**(1):79-83.

Hooper, A., Sebesteny, A. 1973. Variation in *Pasteurella pneumotropica*. *Journal of Medical Microbiology* **7**(1):137-140.

Jakab, G.J., Dick, E.C. 1973. Synergistic effect in viral-bacterial infection: combined infection of the murine respiratory tract with Sendai virus and *Pasteurella pneumotropica. Infection and Immunity* **8**(5):762-768.

Jawetz, E. 1950. A pneumotropic Pasteurella of laboratory animals; I. Bacteriological and serological characteristics of the organism. *Journal of Infectious Diseases* **86**(2):172-183.

Kawamoto, E., Okiyama, E., Sasaki, H., Sawada, T., Mikazuki, K., Ueshiba, H. 2007. Ultrastructural characteristics of the external surfaces of *Pasteurella pneumotropica* from mice and *Pasteurella multocida* from rabbits. *Laboratory Animals* **41**(2):285-291.

Kodjo, A., Villard, L., Veillet, F., Escande, F., Borges, E., Maurin, F., Bonnod, J., Richard, Y. 1999. Identification by 16S rDNA fragment amplification and determination of genetic

diversity by random amplified polymorphic DNA analysis of *Pasteurella pneumotropica* isolated from laboratory rodents. *Laboratory Animal Science* **49**(1):49-53.

Korczak, B., Christensen, H., Emler, S., Frey, J., Kuhnert, P. 2004. Phylogeny of the family *Pasteurellaceae* based on *rpoB* sequences. *International Journal Of Systematic and Evolutionary Microbiology* **54**(Pt 4):1393-1399.

Kunstyr, I., Hartmann, D. 1983. *Pasteurella pneumotropica* and the prevalence of the AHP (*Actinobacillus, Haemophilus, Pasteurella*)-group in laboratory animals. *Laboratory Animals* **17**(2):156-160.

Kwok, S., Kellogg, D.E, McKinney, N., Spasic, D., Goda, L., Levenson, C., Sninsky, J.J. 1990. Effects of primer-template mismatches on the polymerase chain reaction: Human immunodeficiency virus type I model studies. *Nucleic Acids Research* **18**(4):999-1005.

Lane, D.J. 1991. 16S/23S rRNA sequencing. In: Nucleic Acid Techniques in Bacterial Systematics (Eds. E. Stackebrandt & M. Goodfellow), Wiley, Chichester, UK. pp. 115-175.

Laubach, H.E., Kocan, A.A., Satain, K.E. 1978. Lung lysophospholipase activity in specific pathogen free rats infected with *Pasteurella pneumotropica* or *Mycoplasma pulmonis*. *Infection and Immunity* **22**(1):295-297.

Macy, J.D. Jr, Weir, E.C., Compton, S.R., Shlomchik, M.J., Brownstein, D.G. 2000. Dual infection with *Pneumocystis carinii* and *Pasteurella pneumotropica* in B cell-deficient mice: diagnosis and therapy. *Comparative Medicine* **50**(1):49-55.

Mannheim, W. 1984. *Pasteurellaceae* Pohl 1979. In: Bergey's Manual of Systematic Bacteriology. Vol.1 (Eds. Krieg, N.R., Holt, J.G.), Williams & Wilkins, Baltimore. pp. 550-575.

Manning, P.J., Gaibor, J., Delong, D., Gunther, R. 1989. Enzyme-linked immunosorbent assay and immunoblot analysis of the immunoglobulin G response to whole-cell and

lipooligosaccharide antigens of *Pasteurella pneumotropica* in laboratory mice with latent Pasteurellosis. *Journal of Clinical Microbiology* **27**(10):2190-2194.

Matsumiya, L.C., Lavoie, C. 2003. An outbreak of *Pasteurella pneumotropica* in genetically modified mice: treatment and elimination. *Contemporary Topics in Laboratory Animal Science* **42**(2):26-28.

McKeel, R., Douris, N., Foley. P.L., Feldman, S.H. 2002. Comparison of an *espB* gene fecal Polymerase Chain Reaction assay with bacteriologic isolation for detection of *Citrobacter rodentium* infection in mice. *Comparative Medicine* **52**(5):439-444.

Mikasuki, K., Hirasawa, T., Chiba, H., Takahashi, K., Sakai, Y., Ohhara, S., Nenui, H. 1994. Colonization pattern of *Pasteurella pneumotropica* in mice with latent pasteurellosis. *Jikken Dobutsu* **43**(3):375-379.

Monteiro, L., Bonnemaison, D., Vekris, A., Petry, K.G, Bonnet, J., Vidal, R., Cabrita, J., Mégraud. 1997. Complex polysaccharides as PCR inhibitors in feces: Helicobacter pillory model. *Journal of Clinical Microbiology* **35**(4):995-998.

Mutters, R., Ihm, P., Pohl, S., Frederiksen, W., Mannheim, W. 1985. Reclassification of the genus Pasteurella Trevisan 1887 on the basis of deoxyribonucleic acid homology, with proposals for the new species *Pasteurella dagmatis*, *Pasteurella canis*, *Pasteurella stomatis*, *Pasteurella anatis*, and *Pasteurella langaa*. *International Journal of Systematic Bacteriology* **35**(3):309-322.

Nakawaga, M., Saito, M., Kohjima, K. 1981. Mutual transmition of *Pasteurella pneumotropica* between mice and rats. *Nippon Juigako Zasshi* **43**(6):937-940.

Nakagawa, M., Saito, M., Suzuki, E., Nakayama, K., Matsubara, J., Muto, T. 1984. Ten years-long survey on pathogen status of mouse and rat breeding colonies. *Experimental Animals* **33**(1):115-120.

Needham, J.R., Cooper, J.E. 1975. An eye infection in laboratory mice associated with *Pasteurella pneumotropica*. *Laboratory Animals* **9**(3):197-200.

Nicholas, K.B., Nicholas H.B. Jr., Deerfield, D.W. II. 1997. GeneDoc: Analysis and Visualization of Genetic Variation. *EMBNEW.NEWS* **4**:14.

Nicklas, W., Baneux, P., Boot, R., Decelle, T., Deeny, A.A., Fumanelli, M., Illgen-Wilcke, B.; FELASA Working Group on Health Monitoring of Rodent and Rabbit Colonies. 2002. Recommendations for the health monitoring of rodent and rabbit colonies in breeding and experimental units. *Laboratory Animals* **36**(1):20-42.

Nicklas, W. 2007. *Pasteurellaceae*. In: The mouse in biomedical research, Volume 2 – Diseases (Eds. Fox, J.G., Barthold, S.W., Davisson, M.T., Newcomer, C.E., Quimby, F.W., Smith, A.W.), Elsevier, San Diego. pp. 469-505.

Nicklas, W. 2008. International harmonization of health monitoring. *Ilar Journal* **49**(3):338-346.

Nozu, R., Goto, K., Ohashi, H., Takakura, A., Itoh, T. 1999. Evaluation of PCR as a means of identification of *Pasteurella pneumotropica*. *Experimental Animals* **48**(1):51-54.

Olsen, I., Dewhirst, F.E., Paster, B.J., Busse, H.J. 2005. *Pasteurellaceae*. In: Bergey's Manual of Systematic Bacteriology. Vol.2, Part B (Eds. Brenner, D.J., Krieg, N.R., Staley, J.T.), Springer, New York. pp. 851-912.

Otto, G., Franklin, C.L. 2006. Medical Management and Diagnostic Approaches. In: The Laboratory Rat (Eds. M.A. Suckow, S.H. Weisbroth, C.L. Fraklin), Elsevier Academic Press, London. pp.548-564.

Patten Jr, C.C., Myles, M.H., Franklin, C.L., Livingston, R.S. 2010. Perturbations in cytokine gene expression after inoculation of C57BL/6 mice with *Pasteurella pneumotropica*. *Comparative Medicine* **60**(1):18-24.

Pritchett-Corning, K.R., Cosentino, J., Clifford, C.B. 2009. Contemporary prevalence of infectious agents in laboratory mice and rats. *Laboratory Animals* **43**(2):163-173.

Sasaki, H., Kawamoto, E., Okiyama, E., Ueshiba, H., Mikazuki, K., Amao, H., Sawada T. 2006a. Molecular typing of *Pasteurella pneumotropica* isolated from rodents by amplified 16S ribosomal DNA restriction analysis and pulsed-field gel electrophoresis. *Microbiology and Immunology* **50**(4):265-272.

Sasaki, H., Kawamoto, E., Ueshiba, H., Amao, H., Sawada, T. 2006b. Phylogenetic relationship of *Pasteurella pneumotropica* isolates from laboratory rodents based on 16S rDNA sequence. *Journal of Veterinary Medical Science* **68**(6):639-41.

Sasaki, H., Kawamoto, E., Kunita, S., Yagami, K. 2007. Comparison of the in vitro susceptibility of rodent isolates of *Pseudomonas aeruginosa* and *Pasteurella pneumotropica* to enrofloxacin. *Journal of Veterinary Diagnostic Investigation* **19**(5):557-560.

Sasaki, H., Kawamoto, E., Tanaka, Y., Sawada, T., Kunita, S., Yagami, K. 2009a. Comparative analysis of *Pasteurella pneumotropica* isolates from laboratory mice and rats. *Antonie Van Leeuwenhoek* **95**(4):311-317.

Sasaki, H., Kawamoto, E., Tanaka, Y., Sawada, T., Kunita, S., Yagami, K. 2009b. Identification and characterization of hemolysin-like proteins similar to RTX toxin in *Pasteurella pneumotropica*. *Journal of Bacteriology* **191**(11):3698-3705.

Sasaki, H., Ishikawa, H., Sato, T., Sekiguchi, S., Amao, H., Kawamoto, E., Matsumoto, T., Shirama, K. 2011. Molecular and virulence characteristics of an outer membrane-associated RTX exoprotein in *Pasteurella pneumotropica*. *BMC Microbiology* **11**:55.

Scharmann, W., Heller, A. 2001. Survival and transmissibility of *Pasteurella pneumotropica*. *Laboratory Animals* **35**(2):163-166.

Steffen, E.K., Nicklas, W. 1999. Bacteriological and molecular procedures for the detection and characterization of rodent *Pasteurellaceae*. *ACLAD newsletter* [Online], 20(1). Available: http://www.aclad.org/20_1.html [March 15, 2007].

Ueno, Y., Shimizu, R., Nozu, R., Takahashi, S., Yamamoto, M., Sugiyama, F., Takakura, A., Itoh, T., Yagami K. 2002. Elimination of *Pasteurella pneumotropica* from a contaminated mouse colony by oral administration of Enrofloxacin. *Experimental Animals* **51**(4):401-405.

Wang, R.F., Campbell, W., Cao, W.W., Summage, C., Steele, R.S., Cerniglia, C.E. 1996. Detection of *Pasteurella pneumotropica* in laboratory mice and rats by polymerase chain reaction. *Laboratory Animal Science* **46**(1):81-85.

Ward, G.E., Moffatt, R., Olfert, E. 1978. Abortion in mice associated with *Pasteurella pneumotropica. Journal of Clinical Microbiology* **8**(2):177-180.

Weigler, B.J., Wiltron, L.A., Hancock, S.I., Thigpen, J.E., Goelz, M.F., Forsythe, D.B. 1998. Further evaluation of a diagnostic polymerase chain reaction assay for *Pasteurella pneumotropica*. *Laboratory Animal Science* **48**(2):193-196.

Yamada, S., Baba, E., Arakawa, A. 1983. Proliferation of Pasteurella pneumotropica at oestrus in the vagina of rats. Laboratory Animals 17(4):216-266.

Zenner, L., Regnault, J.P. 1999/2000. A retrospective study of the microbiological and parasitological status of laboratory rodents in France. *Journal of Experimental Animal Science* **40**(1-3):211-222.