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TÍTULO: IDENTIFICATION OF BACTERIAL ZOONOTIC RESPIRATORY PATHOGENS IN GUINEA PIGS (*Cavia porcellus*) RAISED AS LIVESTOCK IN PAUTE, ECUADOR

Trabajo de titulación presentado como requisito para la obtención del título de Biólogo

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Dedico el presente trabajo de titulación a toda mi familia, especialmente a mis padres Miguel Zambrano y María Mila por ser mi inspiración y fortaleza durante cada etapa de mi formación académica, y a mi hermana Mishell Zambrano por su apoyo incondicional y confianza.

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ABSTRACT

Bacteria such as *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Mycobacterium tuberculosis* and *Staphylococcus aureus* are among the most common human respiratory pathogens causing tuberculosis, pneumonia, sepsis, and meningitis. *Cavia porcellus*, known as guinea pig, is an animal used as research model for infectious diseases caused by viruses and bacteria. However, there are only few reports addressing its potential as a zoonotic vector in areas where it is raised as livestock, such as in Ecuador, where its population is estimated to be greater than 20 million. In this study, nasopharyngeal carriage of *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Staphylococcus aureus* and the infection with *Mycobacterium tuberculosis*, were evaluated in 80 guinea pigs from six farms in the Azuay province in Ecuador. For all the pathogens tested, 24 animals (30%) carried *Staphylococcus aureus* and 7 animals (9%) carried *Streptococcus spp*. No *M. tuberculosis* infection was detected and no *H. influenzae* was isolated. The *S. aureus* isolates were tested for methicillin resistance and virulence factors. We found six positive MRSA (Methicillin-Resistance-*S. aureus*) and three isolates were positive for Panton-Valentine Leukocidin (*luk-PV*) gene. Our results suggest the potential of guinea pigs as zoonotic vector for MRSA and *Streptococcus spp.* and claim for the implementation of public health policies for a safe production, management and consumption.

Keywords

Streptococcus, *Haemophilus influenzae*, *Mycobacterium tuberculosis*, *Staphylococcus aureus*, *Cavia porcellus*, guinea pig, zoonosis, respiratory infections.

RESUMEN

Bacterias como *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Mycobacterium tuberculosis* y *Staphylococcus aureus* están entre los patógenos respiratorios humanos más comunes causantes de tuberculosis, neumonía, sepsis y meningitis. *Cavia porcellus*, conocido como conejillo de indias, es un animal utilizado como modelo de investigación para enfermedades infecciosas causadas por virus y bacterias. Sin embargo, hay pocos informes que aborden su potencial como vector zoonótico en áreas donde se cría para el consumo humano, como en Ecuador, donde se estima que su población es mayor de 20 millones. En este estudio, se evaluó la condición de portador en la nasofaringe de *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Staphylococcus aureus* y la infección por *Mycobacterium tuberculosis*, en 80 conejillos de indias de seis granjas en Azuay, Ecuador. Con respecto a los patógenos, 24 animales (30%) portaban *Staphylococcus aureus* y 7 animales (9%) portaban *Streptococcus spp*. No se detectó infección por *M. tuberculosis* y no se aisló *H. influenzae*. En los aislamientos de *S. aureus, se* evaluó la resistencia a meticilina y factores de virulencia. Se encontró seis aislamientos positivos de MRSA (*S. aureus* resistentes a meticilina) y tres aislamientos fueron positivos para el gen que codifica la leucocidina de Panton-Valentine (l*uk-PV).* Nuestros resultados sugieren el potencial de los conejillos de indias como vector zoonótico para MRSA y *Streptococcus spp*. y claman la implementación de políticas de salud pública para una producción, gestión y consumo seguros.

Palabras Clave: *Streptococcus*, *Haemophilus influenzae*, *Mycobacterium tuberculosis*, *Staphylococcus aureus, Cavia porcellus*, conejillo de indias, zoonosis, infecciones respiratorias

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INTRODUCTION-JUSTIFICATION

Respiratory infections: a public health problem and its impact in developing countries.

Respiratory infections, are a leading cause of mortality and morbidity worldwide, resulting in over 3 million deaths in 2015, including 2 million of children under five years old largely in developing regions (Rowlinson et al., 2017). Respiratory tract infections include cold, otitis media, influenza-like illness, croup, bronchiolitis, and pneumonia, and hence they involve a wide range of infection-associated diseases affecting the upper as well as the lower respiratory tracts, and whose etiologic agent could be either viral or bacterial pathogen (Stellrecht, 2017).

Even though viral agents account for around 90% of upper respiratory tract infections, and around 30% of lower respiratory tract infections (Korsman, van Zy, Nutt, Andersson, & Preiser, 2012), the respiratory tract can be susceptible to a wide spectrum of non-viral infectious pathogens as well, including different Gram negative and Gram positive bacteria. In fact, diverse bacterial communities including commensals and pathobionts such as *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Moraxella catarrhalis*, and *Staphylococcus aureus* inhabit the respiratory tract mucosa and may occasionally cause infectious diseases (Bosch, Biesbroek, Trzcinski, Sanders, & Bogaert, 2013). From an evolutionary point of view, this latter one reveals a clear example of co-evolution processes between microbial communities and hosts over a period of millions of years (Man, de Steenhuijsen Piters, & Bogaert, 2017).

The infection is largely a complex bacteria-host process, in which the colonization of the nasopharyngeal niche is the first step. In humans, the establishment of nasopharyngeal flora (including *S. pneumoniae, H. influenzae,* and *M. catarrhalis*) occurs during the first weeks of life (Bogaert, De Groot, & Hermans, 2004). This multistage dynamic process involves: acquisition and removal of species, interactions among microorganisms as well as between microorganism and the vertebrate host, and the interference of environmental factors. In steady state, this complex ecosystem essentially plays some beneficial roles for the host since commensal microbiota can contribute to nutrient absorption, provide obstruction and inhibition of other pathogens, and assist in the development and correct performance of the immune system (Söderholm, 2012). However, when this balanced state is disrupted, an overgrowth and invasion by bacterial pathogens may take place, resulting in invasive and non-invasive respiratory diseases, particularly in children and people having a dysfunctional immune system (Bosch et al., 2013).

Bacterial agents causing respiratory infections

Streptococcus pneumoniae

It belongs to the genus *Streptococcus* and the family *Streptococcaceae* (Hardie & Whiley, 1995). This genus covers a wide spectrum of diverse and potentially zoonotic pathogens. It consists of an extensive and diverse group of pathogenic and commensal bacteria, that display extraordinary adaptability to novel hosts, and an unexpected capacity to acquire resistance to drug and immune response (Clewell, 1981). *Streptococcus pneumoniae* is an encapsulated Gram-positive diplococcus and lanceolate-shaped bacterium whose diameter ranges from 0.5 to 1.25 µm. It is a microaerophilic bacterium (growing in low-oxygen concentrations), negative for catalase, and sensible to bile acids (Dion & Ashurst, 2018). *Streptococcus pneumoniae* is the major human pathogen responsible for millions of deaths each year worldwide**,** being considered a causing agent of community-acquired infections in children. *S. pneumoniae* can be the causing agent of both invasive diseases such as bacteremia, septicaemia, osteomyelitis, septic arthritis, pneumonia and meningitis, and non-invasive diseases including bronchitis, otitis media, and sinusitis.

The assumption of *S. pneumoniae* as strictly human pathogen is well known and persists up to this date. However, animals can also be infected and then used as models for studying its mechanisms of pathogenesis, drug testing, and vaccine candidates, and for describing the microbial and host-specific factors for *S. pneumoniae* infection (Chiavolini, Pozzi, & Ricci, 2008). For instance, murine models have been purposed as target animal models to test vaccination (Sabirov & Metzger, 2008). Additionally, there are well-established murine models for studying diverse diseases such as Pneumococcus-associated pneumonia, meningoencephalitis, and otitis caused by pneumococcus.

S. pneumoniae has a wide spectrum of colonization and virulence factors such as a capsule made of polysaccharides, surface proteins and enzymes, and the toxin pneumolysin (Mitchell & Mitchell, 2010). The polysaccharides of the capsule are responsible for raising the host humoral-immune response due to their antigenicity, and promote the production of serotype-specific antibodies by the host. In this context, the antigenic variability present in the different strains has allowed their clustering into 46 serogroups and 93 serotypes (Geno et al., 2015). According to the literature, serotypes 1, 4, 5, 7F, 8, 12F, 14, 18C and 19A are more likely to cause invasive pneumococcal disease in children younger than 5 years of age worldwide (Song, Nahm, & Moseley, 2013).

Haemophilus influenzae

The genus *Haemophilus* belongs to the bacterial family *Pasteurellaceae*, and consists of a group of human-affecting pathogens as well as bacteria affecting a variety of mammalian and avian species (Grebe & Hinz, 1975; Rayan, Flournoy, & Cahill, 1987; Slee & Stephens, 1985). Human-selective *Haemophilus* species, which are obligate parasites, colonize the mucous membranes along the upper respiratory tract and oral cavity, although isolates colonizing the vagina and intestinal tract have been also reported (Kilian, 2015). Many *Haemophilus* species, which have been reported as strictly human-affecting commensals and pathogens including *H. influenzae, H. aegyptius, H. haemolyticus, H. ducreyi, H. parainfluenzae, H. parahaemolyticus, H. paraphrohaemolyticus, H. aphrophilus, H. paraphrophilus* and *H. segnis,* represent around 10% of the bacterial flora along the upper respiratory tract (Kilian, 2015).

Haemophilus influenzae, whose morphology is coccobacillus or small rod (0.3–0.5 × 0.5–3.0 μm), is Gram-negative and oxidase positive. This human-specific pathogen is a facultative anaerobe, requiring nicotinamide-adenine-dinucleotide (factor V) and a source of hemin (factor X) provided in blood agar for *in vitro* growth. Based on their polysaccharide capsule, two strain types, encapsulated (typeable) and unencapsulated (non-typeable) strains, can be distinguished (Fantini & Yahi, 2015). Regarding encapsulation, these strains can be divided into six (*a* to *f*) serotypes, from which the *b* type presents the highest virulence, thus being the leading causative agent of potentially life-threatening invasive diseases. Additionally, nontypeable strains have been reported to be the causing agents of otitis media, sinusitis, and conjunctivitis, and been related to chronic obstructive lung diseases such as bronchitis, cystic fibrosis, and bronchiectasis (Cardines et al., 2012; Tufvesson, Markstad, Bozovic, Ekberg, & Bjermer, 2017).

H. influenzae frequently triggers infectious episodes along the upper and lower respiratory tracts, including pneumonia in children as well as in adults (High, Fan, & Schwartzman, 2015). It is considered the leading cause of bacterial meningitis in children younger than five years and may be the cause of other life-threatening invasive diseases such as epiglottitis and severe sepsis in both children and adults (Fantini & Yahi, 2015). Additionally, it has been associated to nosocomial infections, including ventilator-associated pneumonia (Simberkoff, 2012). Even though its pathogenesis is still poorly understood, it is known that *H. influenzae* has a series of antigenicity and virulence factors such as the polysaccharide capsule, proteins of the outer membrane, lipopolysaccharides, IgA proteases, adhesion proteins, and fimbriae (pili) (Kostyanev & Sechanova, 2012).

Staphylococcus aureus

The genus *Staphylococcus* comprises Gram-positive cocci with a diameter of 0.5 to 1.5 µm. They can be found alone or clustered into pairs, tetrads, short chains or in grape-bunch-like shapes. The *Staphylococcus* species consist of non-motile, non-encapsulated, facultative anaerobe bacteria. Most of them are characterized by the production of catalases (responsible for the breaking down of hydrogen peroxide into water and oxygen), which distinguish them from the *Streptococcus* and *Enterococcus* (catalase negative) genera.

The genus *Staphylococcus* comprises 32 species, from which at least 16 may be part of the skin microbiota and the mucosal surfaces in humans, although the colonization and infection of different *Staphylococcus* species including *Staphylococcus intermedius* in other mammals and birds have also been documented (Brittingham, Temple, & Duncan, 1988; Gómez et al., 2014; Kishida, Sakoda, Eto, Sunaga, & Kida, 2004; Savini, Catavitello, Bianco, Balbinot, & D'antonio, 2009). Among *Staphylococcus* species which have been found as human-strict bacteria, the most important ones from an epidemiological standpoint are *S. aureus* and *S. lugdunensis.* To a lesser extent *S. epidermidis* and *S. saprophyticus* have been also isolated from the human urinary tract (Cho, Naber, Hacker, & Ziebuhr, 2002; Jordan, Iravani, Richard, & Baer, 1980). Furthermore, *S. aureus* is largely distributed among the primates. Some mastitis cases caused by *S. aureus* have also been reported in sheep and cattle (Lam, Lipman, Schukken, Gaastra, & Brand, 1996; Vasudevan, Nair, Annamalai, & Venkitanarayanan, 2003).

S. aureus is a human commensal and pathogenic microorganism with distinguished two strain types, encapsulated and non-encapsulated, based on their polysaccharide cover. Up to eleven *S. aureus* serotypes have been identified so far with a predominance of the 5 and 8 types, which constitute approximately 85% of the total staphylococcal infections in America and Europe (Havaei et al., 2013). Around 30% of humans are colonized by *S. aureus*, being the leading causing agent of bacteremia and infective endocarditis, osteoarticular, skin and soft tissue infections, particularly pleuropulmonary, and device-related infections (Coates, Moran, & Horsburgh, 2014; Tong, Davis, Eichenberger, Holland, & Fowler, 2015; Wertheim et al., 2005). Finally, clinical syndromes such as epidural abscess, meningitis, toxic shock syndrome, and septic thrombophlebitis have been associated with *S. aureus* (Tong et al., 2015).

The wide spectrum of infectious diseases caused by *S. aureus* is related to virulence factors including exoproteins (such as nucleases, proteases, etc.), which favor surface adhesion, invasion, host-immunity avoidance thus provoking a harmful and toxic impact on the host.

Moreover, virulence factors such as SpA, α-toxin, β-toxin , and Panton-Valentine leukocidin (PVL) have been reported to be responsible for lung tissue injury and inflammation (Bien, Sokolova, & Bozko, 2011). It is worth mentioning that *Staphylococcus aureus* is currently drawing too much research attention because of its large resilience to antibiotics, making Methicillin-resistant *Staphylococcus aureus* (MRSA) not only a major cause of nosocomial infections, but also a widespread cause of community-acquired infections (David & Daum, 2010; Herold et al., 1998; Okuma et al., 2002). From a molecular standpoint the *mecA* gene, which is a fragment within a 21- to 60-kb staphylococcal chromosomal cassette mec (SCCmec), may be responsible for conferring antibiotic resistance, and making β-lactam antibiotics such as penicillins and cephalosporins obsolete as therapeutic tools against these bacterial infections (Wielders, Fluit, Brisse, Verhoef, & Schmitz, 2002). Additionally, phenotypic methicillin resistance conferred by homologous genes such as *mecB*, *mecC* and *mecD* have been also reported in different members of the *Staphylococcaceae* family including *S. aureus* (Becker, Ballhausen, Köck, & Kriegeskorte, 2014; Schwendener, Cotting, & Perreten, 2017).

Mycobacterium tuberculosis

The genus *Mycobacterium* constitutes the unique genus in the family *Mycobacteriaceae*. Mycobacteria are characterized to be Gram-positive aerobes, non-spore formers (excluding *M. marinum*), non-motile, slightly concave-shaped or straight rods (0.2 to 0.6 μm by 1.0 to 10 μm) (Pfyffer, 2015). The number of species belonging to this genus is over 188 including many human pathogens and various other environmental species, which inhabit a diverse spectrum of environments such as water bodies, soil, and metalworking fluids (Gupta, Lo, & Son, 2018). The members of *Mycobacterium* genus have been classified into three major clusters: *Mycobacterium tuberculosis* complex, non-tuberculous mycobacteria and *Mycobacterium leprae* (Rahman et al., 2017).

Several species within this genus are major pathogens for human and non-human species, specially the members of *Mycobacterium tuberculosis* complex, which is a set of genetically related species: *M. tuberculosis, M. canettii, M. africanum, M. microti, M. bovis, M. caprae* and *M. pinnipedii* (Forrellad et al., 2013). Within this complex bacterial cluster, the most well-known species is *M. tuberculosis* ("a pathogen of the mammalian respiratory system") due to its large prevalence in human populations, although nonhuman-animal infections have also been documented. On the other hand, *M. bovis* shows a wide range of host infections, including those in humans, domestic and wild bovines, and goats (Velayati & Farnia, 2017).

According to the Center for Disease Control and Prevention (CDC), 25% of whole human population is infected with Tuberculosis (Tb) (CDC, 2019). In 2017, Tb affected 10 million humans, and around 1.3 million deaths were attributed to Tb-related cases around the world, being a leading cause of mortality in HIV-infected people (CDC, 2019). *Mycobacterium tuberculosis* is a Gram-positive bacterium characterized by slow growth, dormancy, complex cell envelope, intracellular pathogenesis, and genetic homogeneity (i.e., a G+C-rich genome) (Cole et al., 1998). Despite the fact that *M. tuberculosis* largely displays genetic similarity, this pathogen shows a high diversity of biological and epidemiological features associated to different lineages, including transmissibility, fitness, and tendency to acquire drug resistance (Nguyen et al., 2016). Particularly strains of the *Beijing* lineage, which is ubiquitous, are associated with the full-scale spread of multidrug-resistant tuberculosis (Merker et al., 2015).

Guinea pig (*Cavia porcellus*): an infectious disease animal model worldwide.

Cavia porcellus, commonly known as "guinea pig", is a domestic rodent belonging to Family *Cavidae.* It is frequently used in the research related to the study of the human immune system because of its similarities with the human immunological genes (UnitProt, n. d.). *Cavia porcellus* is considered a good animal model for toxicology and vaccine research. In fact, research on guinea pigs allowed the discovery of vitamin C, and it was very important in the development of vaccines against diphtheria, dialysis, antibiotics, and asthma drugs (Animalresearch.info, 2016).

Guinea pigs have been employed to model infectious bacterial diseases such as pulmonary, sexually transmitted, ocular, and gastrointestinal infections, which threaten human health worldwide (Padilla-Carlin, McMurray, & Hickey, 2008). Due to the fact that guinea pigs (like humans) have a high susceptibility to staphylococcal infections, this organism model has been employed to test: methicillin-resistant *S. aureus*, dermonecrotic reactions, disseminated intravascular coagulation, infective endocarditis, effects of nutrition on infection, identification of bacterial factors such as staphopains causing septic shock, burn/surgical wounds, and infections due to device-implantation (Padilla-Carlin et al., 2008). On the other hand, the high susceptibility to infectious processes, analogous symptomatology and pathophysiology, delayed-type hypersensitivity response, and adequate response to standard oral chemotherapies have made guinea pig a suited model organism for primary human tuberculosis. Tuberculosis-infected guinea pigs have been widely used in preclinical research of new drugs and vaccines, different drug delivery methods, and drug safety evaluations. Tuberculosis-infected guinea pigs show lymphadenitis, commonly seen in children, and constitutes an adequate model for testing

the cause-effect relationship between malnutrition and tuberculosis affection (Cegielski & McMurray, 2004).

Regarding *Haemophilus influenzae*, guinea pig as a model organism has been used for preclinical evaluation of vaccines and their immunogenicity (Siber, Anderson, Habafy, & Gupta, 1995). Guinea pigs have been an essential organism not only to characterize the local inflammatory response in acute otitis media (Sato, Kawana, Nonomura, & Nakano, 1999), but also to understand the induction of bronchial hyper-reactivity to histamine (Folkerts & Nijkamp, 1985), to comprehend the mechanisms behind deterioration of the pulmonary beta-adrenergic receptor system caused by *Haemophilus influenza* (Engels, Oosting, & Nijkamp, 1987). With respect to *Streptococcus pneumoniae*, extrapulmonary lesions in guinea pigs have been reported and the 19 serogroup has been isolated and cultured from guinea pig tissues (Nakagawa et al., 1986; Parker, Russel, & De Paoli, 1977). Also in guinea pigs, transmission and multiplication of *Streptococcus pneumoniae* in the respiratory tract is notably enhanced by the co-infection with Sendai virus (Saito, Nakayama, Matsubara, Matsuno, & Nakagawa, 1988).

PROBLEM STATEMENT

Guinea pigs: a pet worldwide but a livestock animal in Andean Region so a potential zoonotic vector of infectious diseases.

Guinea pigs were domesticated 5,000 years ago in South America. The population of *Cavia porcellus* commonly known as "cuy" has been estimated in around 35-65 million distributed among Ecuador, Colombia, Peru, and Bolivia. It can be found from the coast up to 4,500 meters over the sea level (Chauca de Zaldívar, 1997). Since ancestral times up to nowadays, this herbivore rodent has been part of human diet in the Ecuadorian highlands due to the high nutritional value of its meat. The advantage of guinea pig husbandry involves the fact of being an herbivore, having a short reproductive cycle, and their high adaptability to different environments. Ecuador occupies the second place in guinea pig agriculture and livestock production with 21 million animals owing to its increasing demand in rural and urban areas (Calvopiña- Fernández, 2018). It is worth mentioning that around 70% of total guinea pig production is carried out following traditional habits, with no technology and hygiene conditions required for food safety (Ministerio Coordinador de Producción, Empleo y Competitividad, 2012).

Used as pets worldwide, guinea pigs are suspected to transmit bacterial pathogens to humans. As an illustrative example, the CDC (2019) has recently reported outbreaks of *Salmonella enteritidis* infections linked to petting guinea pigs. In this context, the widespread acceptance of guinea pigs both as companion animals, and particularly in the Andean countries as a main source of meat, makes them putative bacterial reservoirs and therefore an emerging zoonotic threat to humans, specially to children at home, and to guinea pig breeders in the hatcheries. In this context, it is necessary to study their possible zoonotic potential, including carriage of respiratory pathogens like *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Staphylococcus aureus*, as well as an infection source of *Mycobacterium tuberculosis.*

OBJECTIVE

General objective

- To study the potential role of guinea pigs as a zoonotic vector for the main bacterial respiratory pathogens.

Specific objectives

- To determine in guinea pigs the nasopharyngeal carriage of *Streptococcus pneumoniae, Haemophilus influenzae, Staphylococcus aureus.*
- To determine in guinea pigs the infection with *Mycobacterium tuberculosis*.

METHODOLOGY

Test Animals and Sampling

A total of 80 guinea pigs from 6 farms in Paute (Ecuador) were sampled during the second semester of 2018. Nasopharyngeal washes were carried out using a sterile saline solution and they were placed into 2X STGG medium (Skim Milk- Tryptone –Glucose- Glycerol) (Equal volumes to keep a 1:1 ratio). The STGG medium, which has been reported as a standard transport medium for recovering *pneumococci* and other nasopharyngeal microflora, contains soybean trypticase broth (10%), skim milk (10%), glucose (10%) and glycerol (10%) (O'Brien, Nohynek, & World Health Organization Pneumococcal Vaccine Trials Carriage Working Group, 2003). Finally, the samples were transported at 4 °C to the lab where they were kept frozen at -80 °C until their processing.

Screening for latent infection caused by *Mycobacterium tuberculosis*

Skin tests (commonly known as PPD tests) were performed with a subcutaneous injection of PPD (5 μg/0.1ml) into a tiny shaved area of the guinea pig's body flank. Twenty-four hours later, the size of allergic skin reaction was measured and recorded in each of 80 animals.

Bacteria Isolation and Identification

Sample processing and microbial culture

The total volume of the STGG medium (1 mL) was centrifuged at 14,000 rpm for 5 minutes. The supernatant was discharged, and a pellet of about 60 µL was withdrawn and used for bacterial culturing. Up to 20 µL of the pellet were inoculated on sheep blood agar plates (5%) with and without 5 µg/ml of gentamicin for *Streptococcus* isolation, on chocolate agar plates for isolating *H. influenzae* and on mannitol salt agar plates for the isolation and identification of *S. aureus*. Sheep blood agar and chocolate agar plates were incubated at 37 °C for 18-24 hours in a capnophilic atmosphere with 5% CO_2. On the other hand, mannitol salt agar plates were incubated under aerobic conditions at 37 °C for 18-24 hours.

Bacterial identification: *Haemophilus influenzae*

Haemophilus influenzae is a fastidious microorganism which grows at 37 °C under 5% CO_2 atmosphere and with strict culture requirements such as the addition of hemin (factor X) and nicotinamide-adenine-dinucleotide NAD (factor V). In this context, the conventional test of X and V factor requirement was used for the identification of *Haemophilus influenzae* (Avendaño & Esquenazi, 2019). Firstly, a dense suspension (approx. 1 McFarland) of bacteria was prepared in a sterile saline solution. Then a swab was dipped into the suspension and was rolled on the surface of nutritional agar plate and disks with the X, V, and XV factors were placed on the agar surface. Finally, the plates were inverted and incubated at 35 °C overnight with CO_2 (Tille, 2015). The result was positive for *H. influenzae* when growth could be observed around the XV factor disk or between both factors V and X. Additionally, the growth factor requirements of different *Haemophilus* species allows their differentiation (see Table 1).

Table 1. **Identification of *Haemophilus* species by their growth requirements**

Organism	Requirement for X factor	Requirement for V factor
H. influenzae	+	+
H. parainfluenzae	–	+
H. haemolyticus	+	+
H. parahaemolyticus	–	+
H. aphrophilus	+	–
H. paraphrophilus	–	–

The data was retrieved and adapted from (Cooke & Slack, 2017).

Bacterial identification: *Streptococcus pneumoniae.*

The identification of *Streptococcus pneumoniae* was done using the "optochin" sensitivity test and the bile solubility test (Organización Panamericana de la Salud e Instituto Nacional Colombia., 2004).

Optochin sensitivity test

The optochin (ethylhydrocupreine hydrochloride) sensitivity test is frequently used for differentiating *S. pneumoniae* from other streptococci. In this test, paper disk (6-mm diameter) impregnated with optochin was directly placed on the surface of sheep blood agar (5%), which had been previously inoculated with isolates suspected of harboring *Streptococcus sp*. The plates were incubated under 5% of CO_2 for 24 hours and were subsequently examined in order to visualize inhibition halo, that is, growth inhibition around the discs. Isolates showing inhibition zones greater than 14 mm were considered as susceptible and the isolate identified as containing *S. pneumoniae* (Organización Panamericana de la Salud e Instituto Nacional Colombia., 2004). In the case of alpha-hemolytic strains whose halo ranged from 9 to 13 mm, a bile solubility test had to be carried out, although some recent studies have reported optochin-resistant *S. pneumoniae* strains (Cortes, Orio, Regueira, Piñas, & Echenique, 2008; Robson, Essengue, Reed, & Horvat, 2007). In this context, all isolates presenting alpha-hemolytic colonies suggestive of the presence of *Streptococcus spp*, were preserved for subsequent molecular analysis.

Bile solubility test

The bile salts have the capacity to lyse *S. pneumoniae* in a selective way. In this process, a dense suspension (approx. 1 McFarland) of bacteria was prepared in a sterile saline solution (0.85%), into which 0.5 mL of sodium deoxycholate was added. The solution was mixed slightly and then heated at 35 °C. The sample-containing tubes were observed after 2 hours. The test turned out positive when turbidity loss was observed in the tube, although partial solubility could also be interpreted as positive (Koneman & Janda, 1997; Organización Panamericana de la Salud e Instituto Nacional Colombia, 2004).

Inmunolex™ *S. pneumoniae* (OMNI)

This is a fast latex agglutination test for detecting 92 serotypes of *S. pneumoniae* directly from culture. The kit contains a flask of latex particles coated with pneumococcal antiserum from rabbit, a positive and a negative control, and 25 reaction cards. A drop of latex suspension was placed near a drop of culture medium in the circumference on the card. The two drops were

rapidly mixed up with a stick and they were spread to coat the whole area of the circumference. Positive outcomes are observed within 10 s while negative results appear after 15 s.

Bacterial identification: *Staphylococcus aureus*

Due to its capacity to ferment mannitol at high salt concentrations, agar medium based on salty mannitol was used for identifying the *Staphylococcus aureus*. The cultured plates were incubated at 37 °C for 24 -72 h (Kateete et al., 2010). The positive result occurs when observed a yellowed-colored halo around the bacterial colonies, which means the presence of microorganisms able to ferment the culture medium.

Additionally, *Staphylococcus aureus* was characterized by DNase activity. Bacteria were cultured and incubated on DNase agar at 37 °C during 24 h. Thereafter, HCl (1N) was poured onto the medium. If white zones around the colonies were observed when removing the excess HCl, a positive DNase activity for those colonies was concluded (Kateete et al., 2010).

Molecular serotyping of *Streptococcus pneumoniae* using Multiplex PCR

Streptococcus pneumoniae colonies were scraped from the agar medium and re-suspended into 200 µl of TE buffer (1XTris-EDTA buffer). Then, the isolates were heated over 95 °C for approximately 1 hour in order to lyse the bacteria. The lysates were finally stored at -80 °C until their processing.

The PCR method allows the detection of the most frequent carriage serotypes (Carvalho et al., 2010). The multiplex PCR (mPCR) method was based on the ordered use of 8 PCR primer pools containing the most frequent disease-causing serotypes (see Table 2).

Table 2. **Multiplex RT-PCR: Serotypes considered in each primer pool**

N° Pool	Serotypes	N° Pool	Serotypes
1	23A, 19E, 19A wzy, 19 nma, 19F wzy, 6	4	22F, 9L/N, 11A, 5, 45
2	16F, 38, 15B/C, 34, 33F, 14	5	17F, 7F/A, 15A, 2F, 23B
3	10A, 35F, 4, 3, 1	6	24A/F, 20, 12F, 29
Triplex	9V, sg18, 23F	7	31, 35B, 7C, 8, 24

Based on the positive specimens found in culture, different multiplex RT-PCRs were performed for serotyping *Streptococcus pneumoniae*. In overall, a preparation of 25 µl PCR mixture was specifically made for each reaction by adding 12.5 µl of PCR Master Mix, 0.25 ml of CPS forward primer (0.025 µM), 0.25 ml of CPS reverse primer (0.025 µM), 2.5 µl of primer mix

(0,5 µM c/primer), 7.5 µl of water, and, 5 µl of DNA lysate. For thermal cycling, the setting was as follows: 94 °C for 4 min, followed by 30 cycles of 45 s at 94 °C, 58 °C for 45 s, and 2 min at 64 °C.

The PCR products and positive controls (known serotypes) were separated in a 2% agarose gel. Electrophoresis was run under a constant voltage of 100 V and a current intensity of 100 mA for 45 min. GeneRuler100 bp DNA ladders were used as molecular size markers. to determine the size of the bands and the related serotype.

Antibiotic resistance and factor-virulence genotyping analysis in *Staphylococcus aureus*

Staphylococcus aureus colonies were scraped from the agar medium and were re-suspended into 200 µl of TE buffer (1XTris-EDTA buffer). Firstly, an enzymatic digestion with lysozyme (0.04 g/ml in 100 ml TE) with brief vortexing every 15 minutes was performed at 37 °C for 45 min. Finally, DNA were extracted and isolated using the High Pure PCR Template Preparation Kit (Roche).

The PCR method detected the presence of *mecA, mecC* and *mecD,* which are deemed to underly methicillin resistance, the virulence factor *luck-PV,* and the *nuc* gene encoding the thermostable nuclease in *S. aureus*. At this point, it should be noted that the multiplex PCR (mPCR) includes a primer pool for *mecA, luck-PV,* and *nuc* gene and *16S rRNA* gene. The amplification of both *nuc* and 16S *rRNA* genes then allowed identify and confirm the samples as being *S. aureus*. The PCRs for *mecC* and *mecD* including *16S rRNA* gene were conducted as individual reactions.

To detect the *luck-PV* and *mecA* genes, a preparation of 25 µl PCR mixture was made specific for each reaction by adding 12.5 µl of PCR Master Mix, 0.75 ml of 16S rRNA forward and reverse primers (10 µM), 0.75 ml of *nuc* gene forward and reverse primers (10 µM), 1.25 of *luck-PV* gene forward and reverse primers (10 µM c/primer), 0.3 µl of *mecA* forward and reverse primers, 1.4 µl of water and, 5 µl of lysate DNA. For the detection of the *mecC* and *mecD* genes, a preparation of 25 µl PCR mixture was made specific for each reaction by adding 12.5 µl of PCR Master Mix, 0.75 ml of *16S rRNA* gene forward and reverse primers (10 µM), 1.5 ml of either *mecC* or *mecD* forward and reverse primers (10 µM), 3 µl of ultra-pure distilled water and, 5 µl of lysate DNA. For thermal cycling, the experimental setting was the following: 94 °C for 2 mi, followed by 35 cycles of 30 s at 94 °C, 58.2 °C for 45 s, and 72 °C for 1 min.

Finally, it was performed the gel electrophoresis with the PCR products including positive controls. Fragments were separated in a 2% agarose gel during 45 min under a constant voltage

of 100 V and a current of 100 mA. GeneRuler100 bp DNA ladders worked out as molecular size markers and the resulting band sizes were compared to them for measuring their size and the associated gene.

RESULTS

A total of 80 guinea pigs from six farms in Paute, Ecuador were sampled. Table 3 summarizes the frequency of bacterial respiratory pathogens isolated from nasopharyngeal wash as well as the results of the infection with *Mycobacterium tuberculosis*. No infection caused by *M. tuberculosis* was detected by PPD test. Neither *Haemophilus influenzae* was isolated and cultured in chocolate agar supplemented with X and V factors.

Table 3. **Frequency of each respiratory pathogen and latent infection of *Mycobacterium tuberculosis* in 80 guinea pigs.**

Location	**Latent infection**	**Specific bacterial pathogens**			
	Mycobacterium tuberculosis N(%)	*Haemophilus influenzae* N(%)	*Streptococcus sp.* N(%)	*Staphylococcus aureus* N(%)	No bacteria detected
Farm 1(n=10)	0(0)	0(0)	**1(10)**	0(0)	**9(90)**
Farm 2 (n=10)	0(0)	0(0)	**2(20)**	0(0)	**8(80)**
Farm 3 (n=10)	0(0)	0(0)	**3(30)**	**5(50)**	**3(30)**
Farm 4 (n=20)	0(0)	0(0)	0(0)	**13(65)**	**7(35)**
Farm 5 (n=10)	0(0)	0(0)	0(0)	**6(60)**	**4(40)**
Farm 6 (n=20)	0(0)	0(0)	**1(5)**	0(0)	**19(95)**

Percentages reflect the pathogens detected within each specific group. Totals can exceed 100% because of the co-infections

Only 7 *streptococci* (1, 2, 3 and 1 strains from Farm 1, Farm 2, Farm 3 and Farm 6 respectively, 9% prevalence) were cultured and isolated on gentamicin-sheep blood agar (see Table 4). These strains were identified as *streptococci* according to standard measurements (i.e. colony morphology, latex agglutination test, and bile solubility) and they were optochin resistant. Additionally, the 8 multiplex PCR reactions for *Streptococcus pneumoniae* serotyping were tested with each of 7 isolates, which turned out negative for all the serotypes tested. As to S*taphylococcus aureus,* 24 strains (5, 13 and 6 from Farm 3, Farm 4 and Farm 5

respectively, 30% prevalence), were cultured and successfully isolated on Mannitol salt agar (see Table 4). A single guinea pig from the Farm 3 showed nasopharyngeal co-colonization with *Streptococcus spp.* and *Staphylococcus aureus* (Table 4).

Table 4. **Carriage of *Streptococcus sp, Staphylococcus aureus,* or both in pathogen-carrying guinea pigs**

Location	**Guinea pig**	**Bacteria pathogens**		
		Streptococcus sp.	***Staphylococcus aureus* (positive for both *nuc* and *16s rRNA)***	**Co-colonization**
Farm 1	A1	+	-	-
Farm 2	B2	+	-	-
	B9	+	-	-
Farm 3	EV2	+	-	-
	EV3	+	-	-
	EV4	-	+	-
	EV5	-	+	-
	EV7	-	+	-
	EV8	-	+	-
	EV9	+	+	+
Farm 4	RR2	-	+	-
	RR3	-	+	-
	RR4	-	+	-
	RR5	-	+	-
	RR6	-	+	-
	RR7	-	+	-
	RR8	-	+	-
	RR9	-	+	-
	RR10	-	+	-
	RR11	-	+	-
	RR12	-	+	-
	RR13	-	+	-
	RR15	-	+	-
Farm 5	RG1	-	+	-
	RG3	-	+	-
	RG4	-	+	-
	RG6	-	+	-
	RG7	-	+	-
	RG10	-	+	-
Farm 6	S1	+	-	-

+: Nasopharyngeal sample was positive for *Staphylococcus aureus, Streptococcus sp*. or both.

The detection of *mecA, nuc, luk-PV, 16S rRNA, mecC* and *mecD* genes for all staphylococcal isolates was performed by amplification from genomic DNA, using multiplex PCR. Our results (see Table 5) revealed that 6 isolates including 2 from Farm 3 and 4 from Farm 4 carried the *mecA* gene, but no isolate carried *mecC* and *mecD* genes. Moreover, only 3 strains from Farm 4 carried out the gene for Panton-Valentine leukocidin. Of note, one *Staphylococcus aureus* isolate from Farm 4 carried both *mecA* and *luk-PV* genes (Table 6).

Table 5. **Frequency of S. *aureus* isolates having resistance genes and virulence factors**

Location	**Isolates**			
	Antibiotic resistance			**Virulence factor**
	***mecA*+ N(%)**	***mecC*+ N(%)**	***mecD*+ N(%)**	***luk-PV gene* N(%)**
Farm 3 (n=7)	2(14)	0(0)	0(0)	0(0)
Farm 4 (n=13)	4(38)	0(0)	0(0)	3(23)

Percentages are reflective of gene presence in *Staphylococcus aureus* isolates within each Farm.

Table 6. **Guinea pigs carry S. *aureus* isolates having resistance and virulence factor genes**

Location	**Guinea Pig Code**	**Antibiotic resistance**			**Virulence Factor**
		mecA	***mecC***	***mecD***	***luk-PV***
Farm 3	EV7	+	-	-	-
	EV8	+	-	-	-
Farm 4	RR4	+	-	-	-
	RR6	-	-	-	+
	RR7	+	-	-	-
	RR8	-	-	-	+
	RR13	+	-	-	+
	RR15	+	-	-	-

+: Nasopharyngeal sample was positive for *Staphylococcus* carrying the gene for antibiotic resistance or virulence factor.

DISCUSSION

Streptococci in guinea pigs.

Streptococci have been considered and classified as facultative pathogens. A vast spectrum of endothermic and poikilothermic animals could harbor at least one streptococcal species, which may act as commensal on skin as well as on inner mucosal surfaces (Fulde & Valentin-Weigand, 2012). *S. zooepidermicus* has been widely considered as part of the nasopharyngeal microbial flora in guinea pigs. Additionally, Okewole et al. (1991) reported infection outbreak inflicted by *Streptococcus pyogenes,* which took place in a colony of 800 Dunkin-Hartley guinea pigs, and Parker et al. (1977) isolated *S. pneumoniae*, most often the 19 type, from extra pulmonary lesions in guinea pigs.

A zoonotic potential of different streptococcal species have been widely reported, including *Streptococcus equi subsp. zooepidemicus, Streptococcus canis, Streptococcus suis, Streptococcus porcinus* and *Streptococcus phocae* (Ginders et al., 2017). Specifically, an infection outbreak caused by *Streptococcus pyogenes* in guinea pigs resulted in deaths, involving breeders, sucklings, and weaners (Okewole et al., 1991). Furthermore, a family cluster with *S. zooepidemicus* infections were linked epidemiologically and genetically to infected guinea pigs (Gruszynski et al., 2015). Our results revealed 7 streptococcal isolates, which were optochin resistant. However, amplification of cps (capsule polysaccharide locus) were not observed and it turned out negative for all serotypes. In addition, it was pending the sequencing of the *16S rRNA* gene in order to identify and clarify the *Streptococcus* species. In this context, breeders and their families from the sampled farms could be at potential risk of *Streptococcus* transmission, which may result in asymptomatic carriage and even life-threatening infections.

Staphylococcus aureus in guinea pigs

In guinea pigs, staphylococcal infections not only have been reported in lab conditions as previously stated but also have widely studied in natural conditions. Miyamoto, Kimura, Hatoya, Aoki, & Ishii (2016) isolated methicillin-resistant *Staphylococcus haemolyticus* from a pet guinea pig. In agreement with our results, Mohana et al. (2015) and Ishihara (1980) detected and isolated *Staphylococcus aureus* from skin lesions, and subcutaneous abscess in *Cavia porcellus*.

Nowadays, antibiotic resistance has become one of the biggest threats to human health system, food security, and country development worldwide. From and epidemiological standpoint, our study revealed worrying data such as that 25 % of the staphylococcal strains isolated from

guinea pigs carried *mecA* gene, which confers antibiotic resistance, especially against β-lactam antibiotics including penicillins and cephalosporins the most extensively available group of antibiotics. In fact, penicillins, including Penicillin G, Penicillin V, amoxicillin, nafcillin, and ampicillin, are currently employed for treating Gram-positive bacteria-caused infections, especially *Staphylococcus* species, *Streptococcus pneumoniae*, etc (Petri, 2015). The most interesting finding in our research was the fact that 4% of the staphylococcal strains isolated from guinea pigs carried both *mecA* gene and gene for Panton-Valentine leucocidin (*Luk-PV)*. This is worrying because the potential association between *Luk-PV* gene-carrying *S. aureus* and the largely mortal, progressive, hemorrhagic, necrotizing pneumonia, which has especially been reported in children and immunocompetent adults (Francis et al., 2005).

Recent evidence has also suggested a potential link between colonization of livestock and *S. aureus* carriage and infectious diseases in humans. In the Netherlands, it has been reported a high prevalence of Methicillin-resistant *Staphylococcus aureus* of clonal ST398 lineage in pigs and their breeders. In addition, the ST398 lineage was detected in samples obtained from pig breeding facilities and in food in Austria. Up to 21 bacterial specimens associated with infectious episodes, have been isolated from people since 2006 (Springer et al., 2009). Weese et al. (2006) reported an outbreak of MRSA skin infections caused by transmission from horse to human in a veterinary clinic. More importantly, to the best of our knowledge the current data shows for the first time the carriage of MRSA in guinea pigs raised as livestock, thus denoting a putative zoonosis risk from *Cavia porcellus* to humans, which may result in non-symptomatic carriage and life-treating infections in breeders and their families.

CONCLUSIONS

The novel methodology used for sampling was successful for recovering part of microbial flora colonizing mucosal surfaces of nasopharyngeal tract in *Cavia porcellus* from six farms in Paute in 2018.

In situ PPD test shows no infection caused by *Mycobacterium tuberculosis* in guinea pigs.

Haemophilus influenzae was not isolated and cultured in chocolate agar supplemented with X and V factors.

7 streptococci (9% prevalence) were cultured on gentamicin-blood agar and they were detected according to standard tests (colony morphology, latex agglutination test, and bile solubility), although these were optochin resistant.

Regarding *Staphylococcus aureus*, 24 strains (prevalence of 30%) were successfully cultured and isolated on Mannitol salt agar, and subsequently identified by PCR amplification of *16s rRNA* gene and *nuc* gene.

6 strains of *Staphylococcus aureus* (25%) carried the *mecA* gene but no isolate carried *mecC* and *mecD* genes.

3 isolates (13%) carried gene encoding for Panton-Valentine leukocidin.

A 4% of the staphylococcal strains isolated from guinea pigs carried both *mecA* gene and gene for Panton-Valentine leucocidin. In this context, MRSA carrying gene for Panton-Valentine leukocidin represents a potential human-life threat due to not only its conferred antibiotic resistance including β-lactam antibiotics but also its association with hemorrhagic and necrotizing pneumonia in children and healthy adults.

Guinea pig breeders as well as their families in Paute could be under a potential zoonotic risk of *Streptococcus spp.* and *Staphylococcus aureus* transmission from guinea pigs, which may result in asymptomatic carriage, and even infectious processes.

RECOMMENDATIONS

16s RNA gene sequencing will be essential in order to identify the *Streptococcus* species isolated from nasopharyngeal wash. Subsequently, a bibliographic analysis will allow to understand their ecological role, possible pathogenicity and zoonotic potential.

It is recommended to recover lung biopsies from symptomatic infected guinea pigs in order to establish the invasive respiratory-infection-causing agents.

It is required to recover samples of the nasopharyngeal mucosa from guinea pig's breeders and to isolate *Staphylococcus aureus* and *Streptococcus spp. in* order to understand a possible zoonotic transmission.

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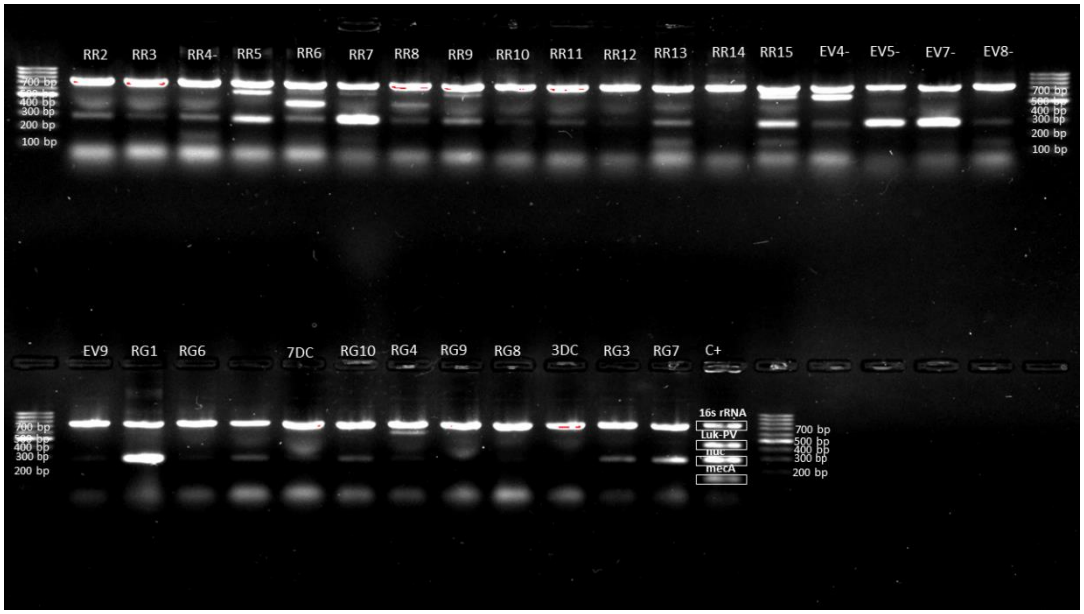
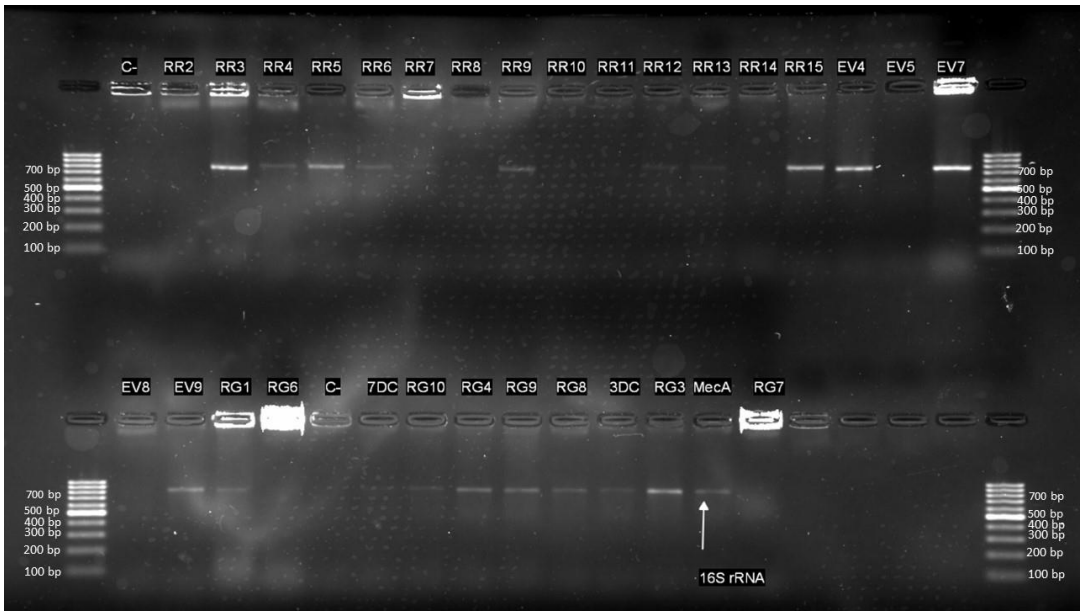
ANNEXES

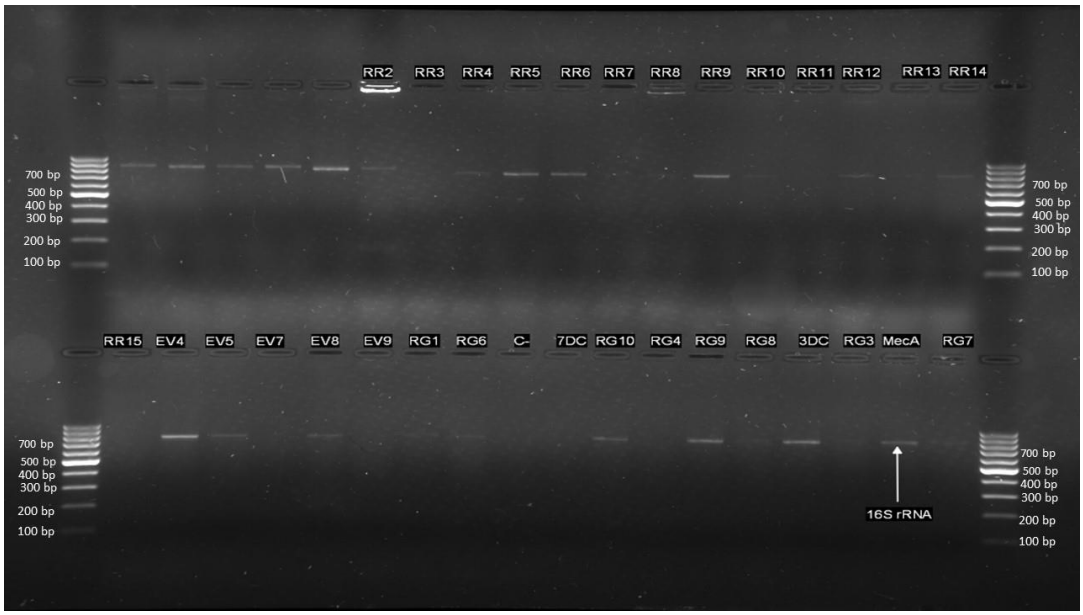
Annex 1. Antibiotic resistance genotyping (*mecA* gene) and factor-virulence genotyping (*nuc* gene and *luk-PV* gene) in *Staphylococcus aureus* isolates.



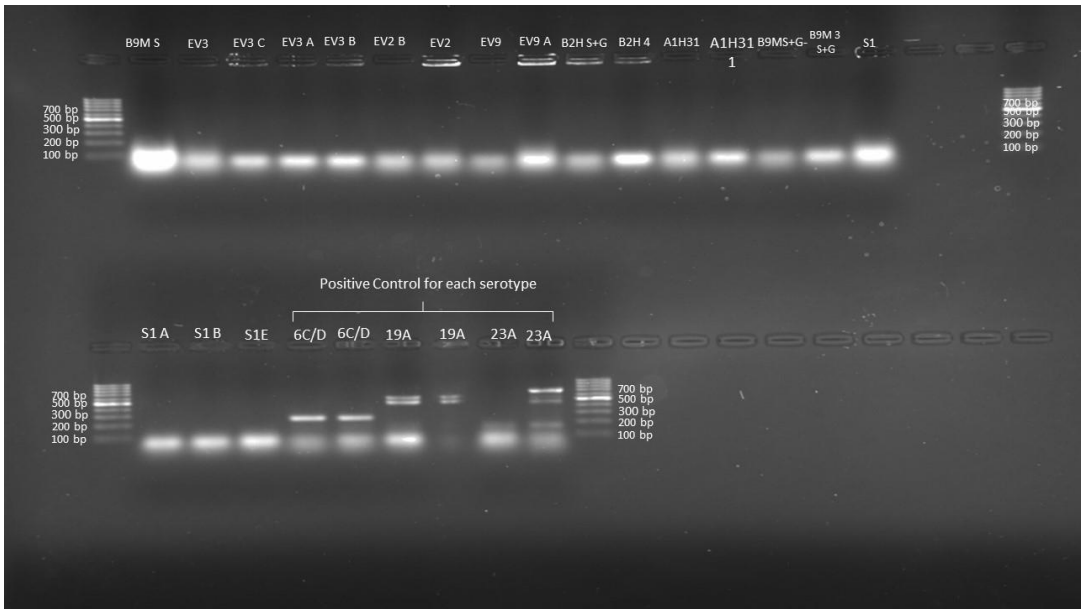
Annex 2. Antibiotic resistance genotyping (*mecC* gene) in *Staphylococcus aureus* isolates.



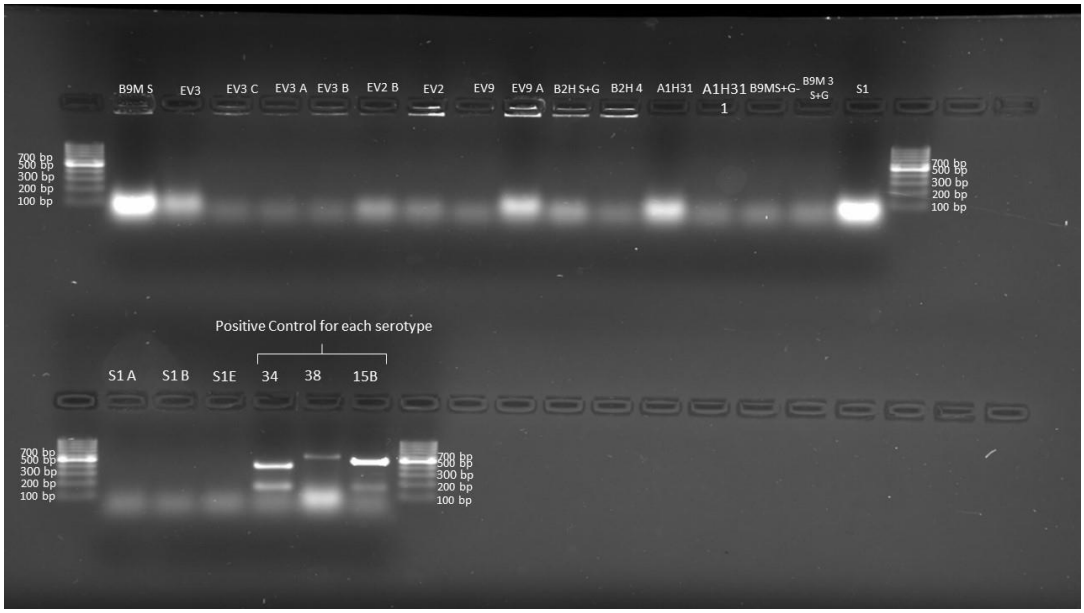
Annex 3. Antibiotic resistance genotyping (mecD gene) in *Staphylococcus aureus* isolates.



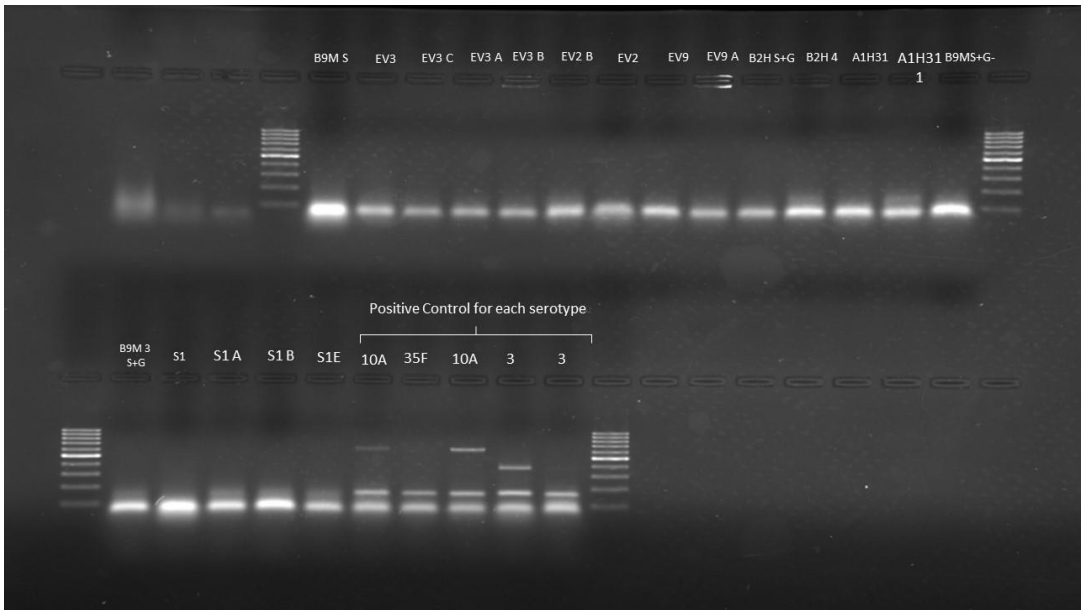
Annex 4. Reaction 1 of *Streptococcus pneumoniae* serotyping on Streptococcal isolates from *Cavia porcellus*.



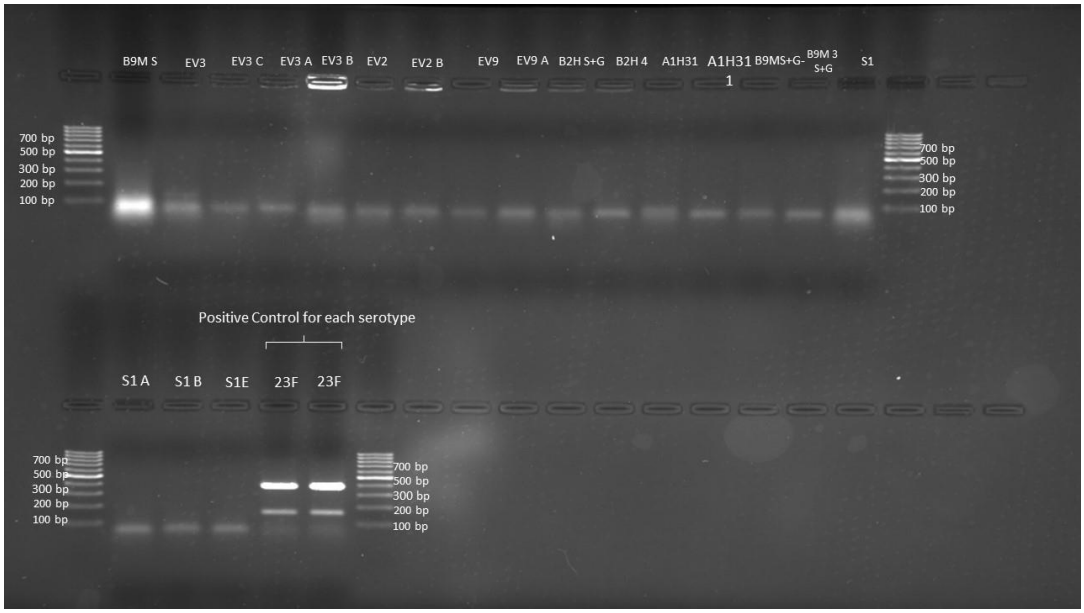
Annex 5. Reaction 2 of *Streptococcus pneumoniae* serotyping on Streptococcal isolates from *Cavia porcellus*.



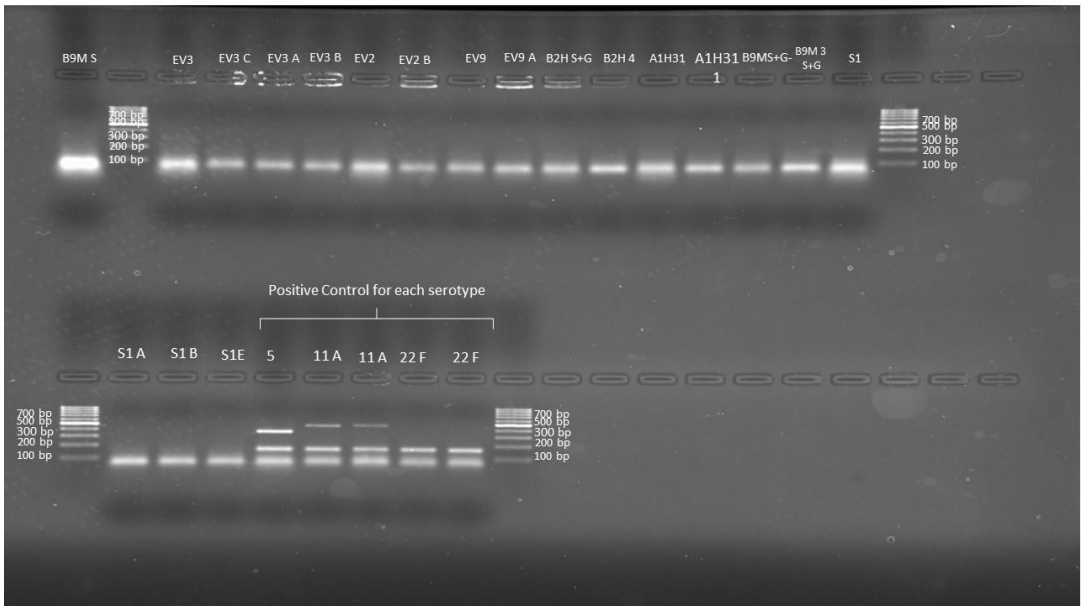
Annex 6. Reaction 3 of *Streptococcus pneumoniae* serotyping on Streptococcal isolates from *Cavia porcellus*.



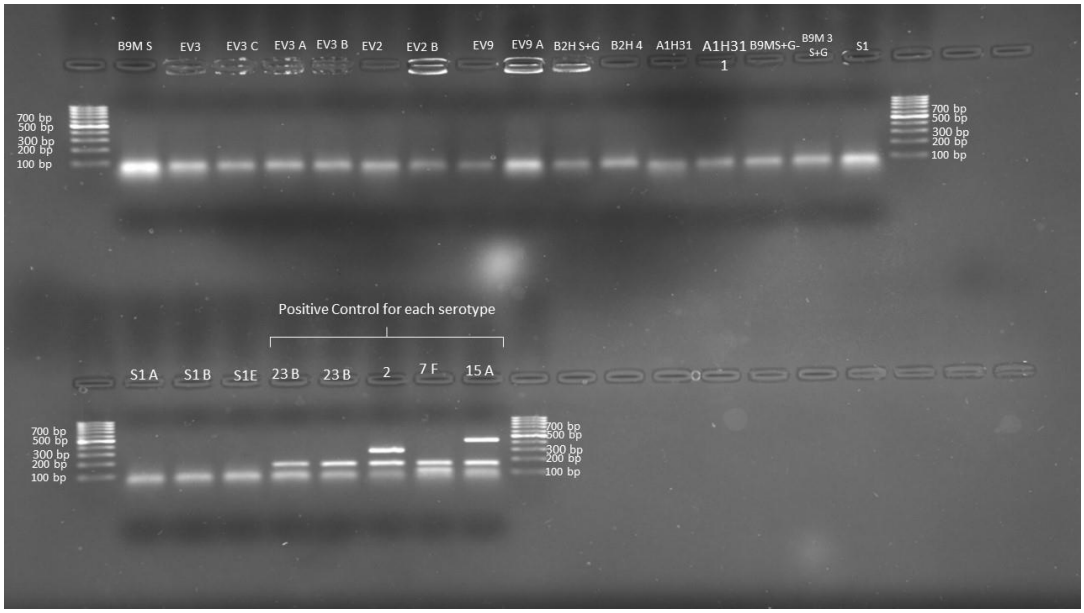
Annex 7. Reaction triplex of *Streptococcus pneumoniae* serotyping on Streptococcal isolates from *Cavia porcellus*.



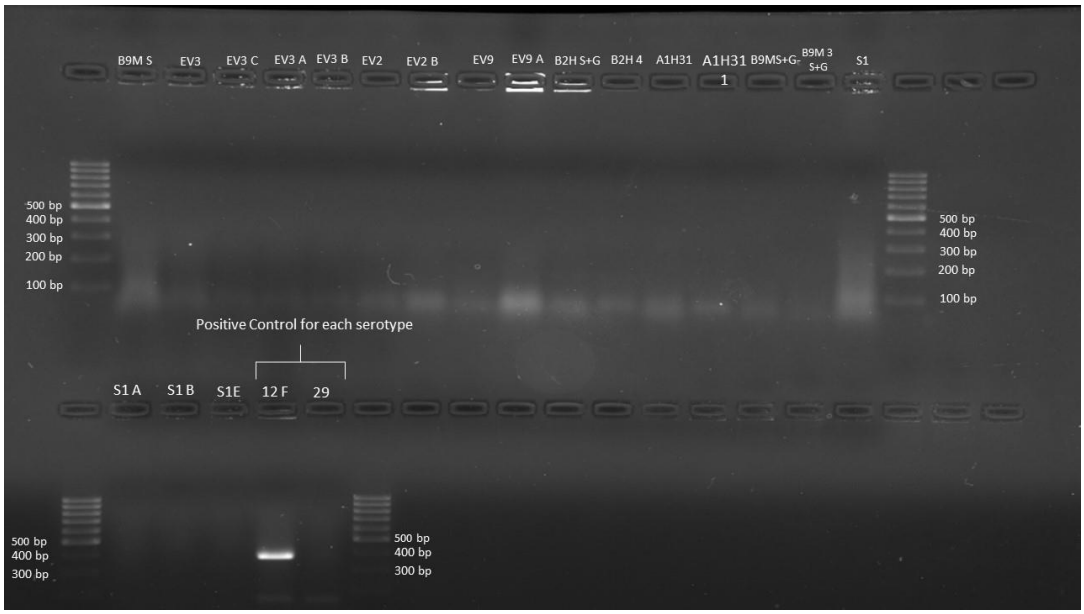
Annex 8. Reaction 4 of *Streptococcus pneumoniae* serotyping on Streptococcal isolates from *Cavia porcellus*.



Annex 9. Reaction 5 of *Streptococcus pneumoniae* serotyping on Streptococcal isolates from *Cavia porcellus*.



Annex 10. Reaction 6 of *Streptococcus pneumoniae* serotyping on Streptococcal isolates from *Cavia porcellus*.



Annex 11. Reaction 7 of *Streptococcus pneumoniae* serotyping on Streptococcal isolates from *Cavia porcellus*.