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First isolation and molecular identification of *Enterobacter hormaechei* in a sheep production unit with respiratory syndrome from Northern Veracruz, Mexico

Primer aislamiento e identificación molecular de *Enterobacter hormaechei* en una unidad de producción de ovinos con presencia de síndrome respiratorio en el Norte de Veracruz, México

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ABSTRACT

Sheep production in Mexico encounters notable challenges, among which infectious diseases stand out as a growing concern. Ovine respiratory syndrome (ORS) is particularly problematic, being a leading cause of economic losses in the industry. However, despite its significant negative impact, there remains a lack of comprehensive understanding regarding the microorganisms involved. For this reason, the aim of this study was to isolate and identify the causal agents responsible for a respiratory disease outbreak in a sheep production unit in Northern Veracruz. This included: collection of samples from affected sheep, focusing on those displaying clinical signs of respiratory illness; microbial culturing and molecular identification of the isolated bacteria. We isolated rounded, raised colonies with a pink mucoid aspect which were molecularly identified as *Enterobacter hormaechei*, which exhibited a similarity of 99.59% with sequences from China. The isolation and molecular identification of *E. hormaechei* provide new insights into the pathogens affecting sheep, highlighting the importance of continuous surveillance and research in improving sheep health and production. This study represents a significant step in identifying and understanding the causal agents of ORS in Northern Veracruz.

Key words: Enterobacteria; emerging pathogen; respiratory complex; small ruminant; Mexico

RESUMEN

La producción ovina en México enfrenta desafíos notables, entre los que destacan las enfermedades infecciosas como una preocupación creciente. El síndrome respiratorio ovino (SRO) es particularmente problemático y es una de las principales causas de pérdidas económicas en la industria. Sin embargo, a pesar de su importante impacto negativo, sigue existiendo una falta de comprensión integral sobre los microorganismos involucrados. Por esta razón, el objetivo de este estudio fue aislar e identificar los agentes causales responsables de un brote de enfermedad respiratoria en una unidad de producción ovina del Norte de Veracruz. Esto incluyó: recolección de muestras de ovejas afectadas, centrándose en aquellas que presentan signos clínicos de enfermedad respiratoria; cultivo microbiano e identificación molecular de las bacterias aisladas. Aislamos colonias redondeadas, elevadas, de aspecto mucoide rosado, que fueron identificadas molecularmente como *Enterobacter hormaechei*, las cuales exhibieron una similitud del 99,59% con secuencias detectadas en China. El aislamiento y la identificación molecular de *E. hormaechei* proporcionan nuevos conocimientos sobre los patógenos que afectan a las ovejas, destacando la importancia de la vigilancia y la investigación continuas para mejorar la salud y la producción de las ovejas. Este estudio representa un paso importante en la identificación y comprensión de los agentes causales de la SRO en el norte de Veracruz.

Palabras clave: Enterobacteria; patógeno emergente; complejo respiratorio; pequeños rumiantes; México

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INTRODUCTION

Sheep (*Ovis aries*) production in Mexico represents one of the four most important sectors in the generation of animal protein for national consumption. It is estimated that there are 5,714,882 sheep nationwide. In fact, Veracruz state is one of the top 10 states in terms of sheep population, accounting for 4.40% of the national production, with 251,322 animals in production units [1]. Particularly, the North of Veracruz concentrates 25% of the state's production, which 62,903 animals.

Sheep national production indeed faces significant challenges, with infectious diseases emerging as a growing problem [2, 3]. Ovine respiratory syndrome (ORS) represents one of the main causes of economic losses because it affects 10% to 40% of sheep, being a leading cause of death [4]. In lambs, this syndrome led to significant adverse effects such as mortality and poor quality of lambs produced [4, 5]. The ORS is caused by a complex interaction of factors, including the presence of opportunistic bacteria (key pathogens involved include *Mannheimia haemolytica*, *Bibersteinia trehalosi*, *Pasteurella multocida*, *Mycoplasma* spp., and *Escherichia coli*, which are typically harmless but can invade the respiratory tract under certain conditions, leading to disease), environmental conditions (particularly, extreme temperature changes and poor ventilation), and inadequate animal husbandry practices (such as overcrowding, weaning time, transport to other facilities, mixing animals from different origins, and coexistence of generational strata) [2, 3, 4, 5, 6].

In the state of Veracruz, there is a lack of information related to the surveillance and molecular typing of microorganisms causing ORS in sheep flocks. For this reason, the aim of the present study was to identify the causal agents of an outbreak of ORS in a sheep production unit in Northern Veracruz, Mexico. By isolating and molecularly characterizing the pathogens involved, this research aims to contribute to a better understanding of the epidemiology and management of respiratory diseases in sheep populations.

MATERIAL AND METHODS

Sampling

The study was conducted in a sheep production unit located in the municipality of Tihuatlan, Veracruz, within the Huasteca Baja region of Northern Veracruz. The municipality's geographical coordinates are 20°43'1" N, 97°32'13" W, and it is situated at an altitude of 105 MASL. Median temperature is 30.7°C, relative humidity about 63–65%, and precipitation of 15.8–16.0 mm. The production unit comprises approximately 500 hair sheep.

Study population

In May 2022, lambs and adult animals in the production unit exhibited respiratory signs such as expectoration, rhinorrhoea, lethargy, and loss of appetite. These symptoms were accompanied by an alarming increase in mortality rates among the sheep. In response to the situation, animals showing signs of illness were quarantined, and further investigation was initiated to identify potential causal agents. A total of 30 sheep were sampled, 22 were females and 8 males, with ages ranging from one month to two years, with an average of 1.5 years.

During the investigation, sheep were physically restrained, and demographic data along with physiological constants (temperature, respiratory rate and heart rate) of each animal were recorded. After physical restraint, the exterior of the nose of each animal was disinfected with Mycrodacyn, and a sterile Dacron®/polyester swab was inserted into the nostril and rotated against the wall of the nasal cavity. Nasal swab samples were collected from the affected animals and preserved in 15 mL conical tubes containing sterile saline to conserve bacterial and mycotic agents. Subsequently, the samples were maintained in a cold chain to preserve their integrity until their arrival at the laboratory for analysis.

Bacterial and mycotic isolation

Samples taken individually streaking nasal swab were inoculated directly onto selective agar media (MacConkey and blood agar for bacterial isolation, and Sabouraud Dextrose Agar, Emmons with Gentamicin for fungal isolation) which were incubated (Ecoshel, 91210, Pharr, USA) at 37°C for 72 hours (h) and checked every 12 h for the presence of microbial growth.

Molecular identification of bacterial isolates

On each solid culture plate with growth, several colonies of interest (with similar morphology, size and coloration) were selected for DNA extraction. They were placed with the help of a microbiological loop in 1.5 mL low-adherence conical tubes (LoBbind). For each tube we added 500 µL of a 10% Chelex 100 chelating resin solution (Bio-Rad®, United States of America (USA) with 20 µL of proteinase K (SIGMA life sciences®, USA) and incubated at 56°C for one hour (IVYX Scientific, 0745556232573, Washington, USA). Subsequently, the temperature was increased to 94°C for 15 min to denature excess of proteins and were centrifuged at 8,000 G (Hsiang Tai Model CN-3600, Taiwan, China) for 15 min. Samples were allowed to cool to room temperature, supernatant were recovered in new tubes and then frozen at -20°C until use (Hisense®, FC88D6BWX1, China). A 450 bp fragment of the 16S ribosomal gene was amplified in a Veriti 96-Well Fast Thermal Cycler (ThermoFisher Scientific, 4375305, Massachusetts,USA). The reaction mix consisted of 12.5 µL of GoTaq® Green Master Mix, 2× Promega Corporation (Madison, WI, USA), 1 µL of each oligonucleotide (EHR01 GCCTAACACATGCAAGTCGAACG and EHR02 GCCCAATAATTCCGAACAACG) [7], 1 µL of DNA (50 ng) and 9.5 µL of nuclease-free water. We followed the thermal PCR conditions previously proposed [7]. PCR products were visualized by electrophoresis (Shelton Scientific®, QS-710, EUA) in 2% agarose gels stained with Midori green and run with 1% TAE running buffer solution at 85V for 40 min. Positive amplicons were sent for sequencing (Applied BiosystemsTM, 3130xl, EUA) to Macrogen, Korea. The sequences obtained in this study were aligned with those of other validated bacterial species from the same genera deposited in GenBank using the Clustal W algorithm in MEGA 10. We selected the nucleotide substitution model based on the lowest AICc (Akaike information criterion, corrected). A phylogenetic reconstruction was generated using Maximum Likelihood, with 1000 Bootstrap replicates, using the close neighbour interchange method. Gaps were excluded from the analysis.

RESULTS AND DISCUSSION

The physiological constants recorded in the 30 sampled animals were as follows: Temperature 39.84 ± 0.75 (39–41) °C, respiratory rate 17.84 ± 2.95 (14–24) BPM, and heart rate 82.68 ± 9.38 (70–97) BPM. A total of 22 positive culture media for bacterial growth were

obtained, which described rounded, raised colonies with a pink mucoid aspect. None of the Sabouraud media were positive for fungal growth. All media with growth were tested for the detection of bacterial using universal 16S rDNA primers. Males exhibited the highest percentage of infection with an 87.5% (7/8), followed by females with 68.18% (15/22), representing a frequence of 73.33% (22/30). However, we only recovered 20 sequences with adequate quality for phylogenetic analysis.

The set of 20 consensus sequences obtained had 490 bp, coverage of 99–100%, and similarities ranging 99.59% with sequences of *Enterobacter hormaechei* from China (GenBank Accession numbers MN007139.1 and KJ660958.1). Sequences generated in this study were deposited in GenBank under the following accession numbers: PP893165–PP893184. The ML reconstruction grouped our sequences in the same branch with other *E. hormaechei* sequences deposited in GenBank supported by a bootstrap value of 63%. (FIG. 1).

Ovine respiratory syndrome is commonly attributed to bacterial agents of the *Pasteurella*, *Mannheimia*, *Bibersteinia*, and *Mycoplasma* genera, the inclusion of *E. hormaechei* among the bacterial agents is notable [2, 4, 5, 6].

The identification of *E. hormaechei* in sheep with respiratory problems marks a significant discovery, particularly as it is the first report of this microorganism in ORS in Mexico and Latin America. This suggests a potential emerging pathogen in sheep populations in the region. *E. hormaechei* is a Gram–negative oxidase–negative, fermentative bacterium and a member of the *Enterobacter cloacae* complex (ECC), is primarily known for its significance in human clinical settings, where it can cause serious nosocomial infections [8]. Its presence in ORS adds a new dimension to our understanding of respiratory diseases in ovine populations [4, 8]. The identification of *E. hormaechei* expands this list and underscores the importance of considering a broad range of potential pathogens in the diagnosis and management of respiratory diseases in sheep.

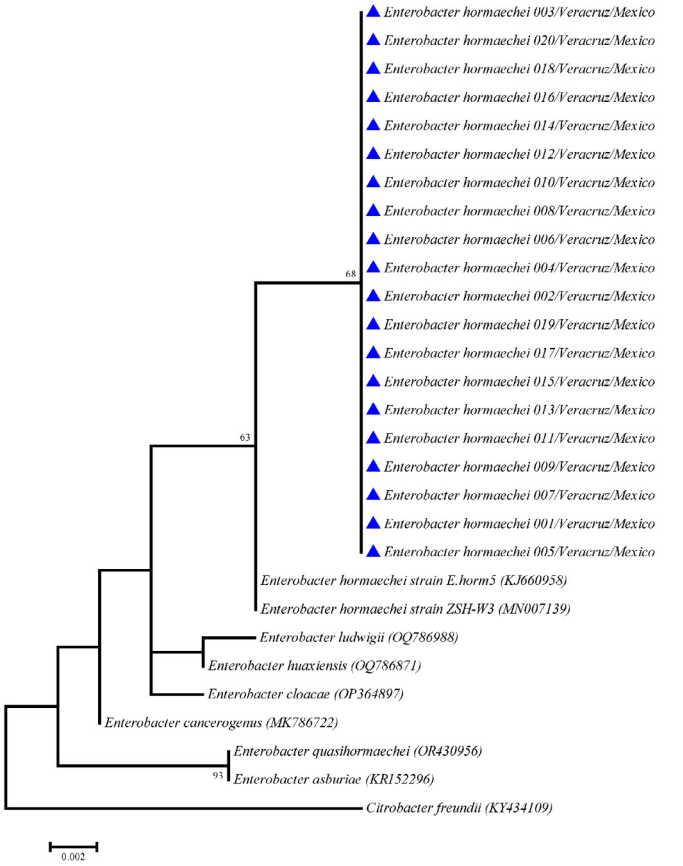

FIGURE 1. Maximum likelihood phylogenetic tree generated using T93 + G+I distance model with partial sequences of the 16S rRNA gene from several members of the genus *Enterobacter*. Bootstrap values greater than 50 are indicated at the nodes. Sequences generated in this study are marked with solid forms

The expanding recognition of *E. hormaechei* as a potential pathogen in various animal species accentuates its importance in veterinary medicine and highlights the need for increased awareness and surveillance of this microorganism. Reports of *E. hormaechei* in different animal species, including pigs, foxes, and cattle, suggest its ability to infect a wide range of hosts and potentially cause various clinical conditions, from diarrhoea in piglets to severe respiratory conditions in calves [9, 10, 11]. The recent identification of *E. hormaechei* as the causative agent of fatal respiratory conditions in calves and lambs in China emphasizes the significance of understanding its role in animal health and disease [8, 9, 10].

Recently, the isolation of *E. hormaechei* from lung tissue samples of sheep, as well as its genetic similarity to strains from cattle, suggests a possible cross–species transmission route which is strengthened by the findings of the present study [8, 9, 10]. In the context of the farm studied in the present study, where dual–purpose cattle are present, but no effective preventive medicine program is in place, the lack of biosecurity measures such as sanitary mats or controlled movement between cattle grazing sites and sheep sheds may facilitate transmission, potentially through fomites. The observation of a high number of animals per square meter, experiencing rapidly evolving respiratory problems leading to increased mortality suggests a significant health challenge within the sheep population on the analysed farm. Several factors may contribute to this situation, like immunosuppression triggered by seasonal changes and decreasing temperatures [4, 5, 6]. Respiratory diseases in sheep are often exacerbated during periods of environmental stress, such as seasonal transitions, which can weaken the animals' immune system and increase their susceptibility to infections [4, 6].

Multiple reports of resistance to a wide range of antibiotic therapies by this species of *Enterobacter* exist [11, 12, 13], particularly to β–lactam and quinolone, which are widely used in the human clinic, which is why this species must be approached from the perspective of comprehensive perspective of One Health to reduce the risk of circulation between species and establishment in humans [10, 12, 13].

Despite regular treatment with enrofloxacin in animals from the studied farm, the persistence of respiratory problems suggests a potential issue with treatment efficacy. The literature indicates that resistance to quinolones, including enrofloxacin, is a growing concern in veterinary medicine, with many strains of bacteria exhibiting resistance to these antibiotics [12, 13].

In this study, the lack of establishment of an antibiotic susceptibility profile for the recovered colonies hinders the ability to identify potential antibiotic resistance. Understanding the susceptibility of bacterial isolates to antibiotics is crucial for guiding appropriate treatment strategies and preventing the spread of resistant strains.

Moving forward, it is essential to conduct a comprehensive antibiotic susceptibility testing on bacterial isolates from affected animals to determine the most effective treatment options. Additionally, apply strategies to minimize the risk of antibiotic resistance, such as prudent antibiotic use, surveillance of resistance patterns, and implementation of infection control practices, is crucial for maintaining animal health and welfare on the farm.

CONCLUSIONS

In this work, the presence of *E. hormaechei* with a frequence of 73.33% was detected for the first time through microbiological isolation and molecular typing in sheep with respiratory symptoms in a livestock farm in the North of Veracruz, which suggests that these bacteria must be monitored as a possible causal agent of ORS in Mexico and other Latin American countries. Continued research is essential to explore the prevalence, impact, and control strategies for *E. hormaechei* and other pathogens in sheep populations.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Ethical approval

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. This study was approved by the Bioethics Committee of the Facultad de Ciencias Biológicas y Agropecuarias, Campus Poza Rica–Tuxpan of the Universidad Veracruzana (UV) (Animals were handled according to National Legislation and Ethics (NOM–012–ZOO–1993).

Data availability

All datasets used and/or analyzed during this study are included in this article. Sequences generated are deposited in GenBank under Accession numbers: PP893165–PP893184.

Consent to participate

Not applicable

Consent for publication

Not applicable

Author contributions

Javier C. Huerta–Peña: Conceptualization, Data curation, Investigation, Methodology, Writing – original draft. Gabriela R. Hernández–Carbajal: Conceptualization, Data curation, Investigation, Methodology, Writing – original draft. Gerardo G. Ballados–González: Investigation, Supervision, Writing – original draft, Writing – review and editing. José M. Martínez–Hernández: Investigation, Supervision, Writing – original draft, Writing – review and editing. José A. Villagómez–Cortés: Investigation, Supervision, Writing – original draft, Writing – review and editing. Sokani Sánchez–Montes: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Project administration, Writing – original draft, and Writing – review and editing.

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