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Bovine leukemia virus detection in humans: A systematic review and meta-analysis

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ABSTRACT

To review the available studies on the frequency of detection of the *bovine leukemia virus* in human samples, a systematic review with meta-analysis of the scientific literature was carried out, including papers published in English, Spanish, and Portuguese in 5 multidisciplinary databases. We collected information from different populations following a detailed and reproducible search protocol in which two researchers verified the inclusion and exclusion criteria. We identified 759 articles, of which only 33 met the inclusion criteria. Analyzed studies reported that the presence of the virus was measured in human samples, such as paraffin-embedded breast tissue and peripheral blood from 10,398 individuals, through serological and molecular techniques. An overall virus frequency of 27% (Ranging between 17 and 37%) was observed, with a high-frequency data heterogeneity between studies. The presence of this virus in different human biological samples suggests the need to investigate further its transmission route to humans and its potential role in developing and progressing diseases.

1. Introduction

Bovine Leukemia Virus (BLV) belongs to the *Deltaretrovirus* genus and *Retroviridae* family; it is closely related to human T-cell lymphotropic virus 1 (HTLV-1) (Pluta et al., 2020). Morphologically, the viral particle has a diameter that ranges between 60 and 125 nm. Like all retroviruses, BLV possesses the genes *gag, pro, pol,* and *env*, which encode the internal structural proteins, viral protease, reverse transcriptase, and envelope glycoproteins of the virion, respectively, which are essential to produce infectious viral particles. Two identical long terminal repeats (LTRs) are flanking these genes (Chameettachal et al., 2023). Moreover, BLV and HTLV-1 have a *pX* sequence between the *env* gene and the 3′LTR region that encodes the regulatory proteins Tax and Rex. In BLV and HTLV-1, the Tax protein acts as an activator of transcription with oncogenic potential, while Rex interferes with the exportation of messenger RNA of both viruses from the nucleus (Derse, 1987; Felber et al., 1989; Willems et al., 1987; Willems et al., 1990).

BLV can naturally infect cattle, capybaras, and sheep. Nevertheless, *in vitro*, BLV can infect different cell types from various species, including humans, such as human lung embryonic cells (WI-38), human tumor (ARH77 and K562), and neural-origin human cells are also highly susceptible to BLV infection (Altaner et al., 1989; Camargos et al., 2004; Delarmelina et al., 2020). For many years, it was believed that humans were not exposed; however, various reports have emerged in the last decades about the presence of BLV in humans for the reason that the identification of different biological markers and gene fragments in the proviral stage (Giovanna et al., 2013; Buehring et al., 2014; Khalilian et al., 2019), proteins (Uribe et al., 2006) and antibodies reactive against the virus as a sample of exposure to this virus (Buehring et al., 2003). It is now known that BLV may be present in the breast (Buehring et al., 2014; Khalilian et al., 2019; Schwingel et al., 2019; Baltzell et al., 2018; Lendez et al., 2018; Buehring et al., 2017; Buehring et al., 2015), lung (Robinson et al., 2016), and blood cells (Buehring et al., 2019) in humans.

Studies published since 1976 demonstrated the ability of BLV to infect various human cell lines. Cell cultures from humans, primates (Chimpanzees and Rhesus monkeys), canines, sheep, goats, and bats were infected with BLV-infected cell lines and by inoculation with cell-

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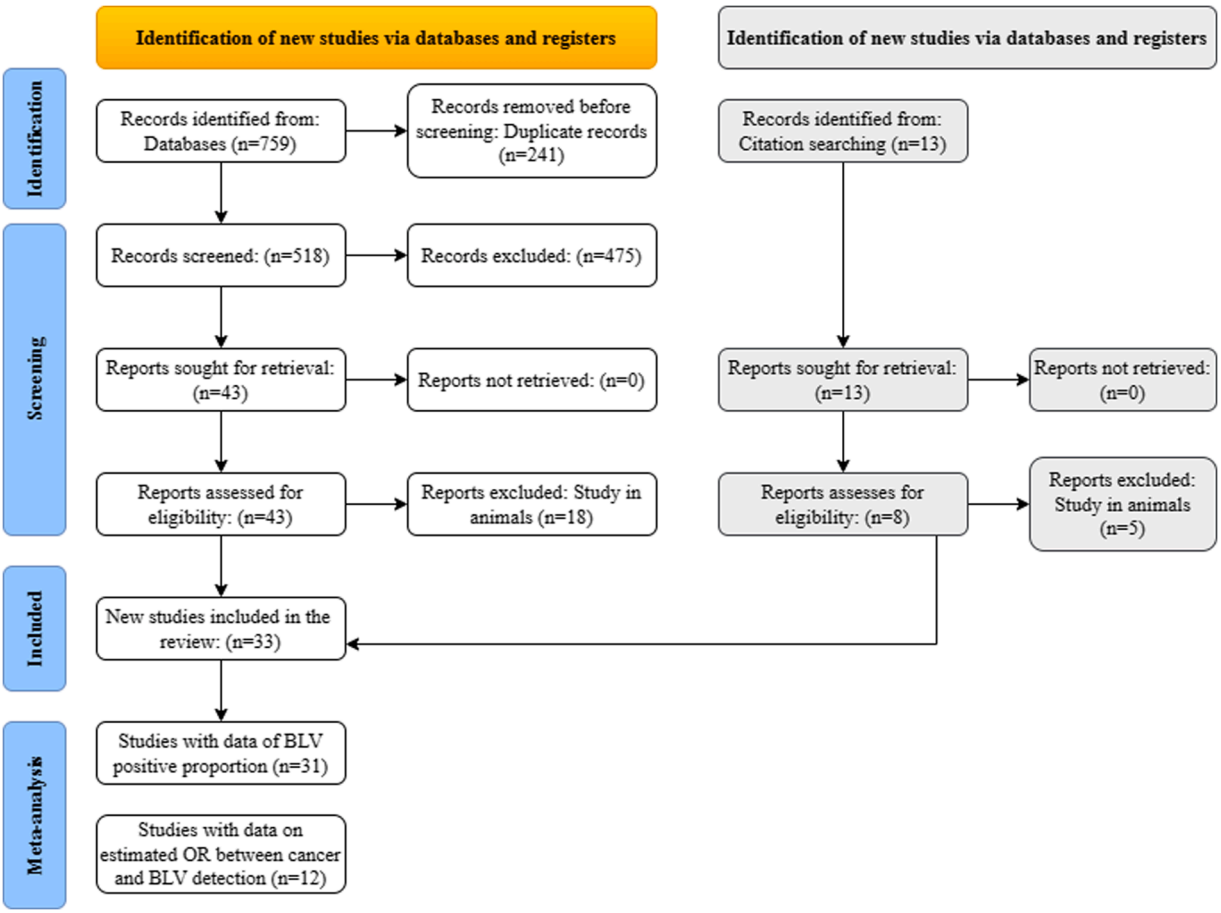

Fig. 1. The flowchart describing the screening process of 33 articles analyzed in this study.

free virus preparations in all cell cultures, the production of the complete virus was observed (Graves and Ferrer, 1976). Several studies report the persistence of BLV in various types of cells (Doménech et al., 2000; Heeney et al., 1992; Rovnak et al., 1991; Aida et al., 1989). However, it has been defined that the primary cellular target of the virus is B lymphocyte that expresses surface immunoglobulin M. In addition to B lymphocytes, BLV also persists in cells of the monocyte/macrophage lineage (Aida et al., 1993; Mirsky et al., 1993). Until now, two proteins involved in cellular transport have been proposed as potential cellular receptors for BLV, AP3D1, and CAT1/SLC7; these proteins are common in mammals, sharing high percentages of identity between species (Corredor et al., 2018; Bai et al., 2019). In the natural host, the infection is transmitted horizontally by transferring infected cells by direct contact, iatrogenic route, and through hematophagous insects (Ferrer, 1978; Gillet et al., 2007). Another transmission route is vertical transmission (cow to calf) across the placenta and colostrum and milk alimentation from infected animals (Ferrer, 1979).

With the evolution of diagnostic methods, various reports have emerged in the last decades about the presence of BLV in humans. Several studies have explored the possible link between BLV and human disease, especially breast cancer, but the data remains controversial (Buehring et al., 2003; Burridge, 1981; Burny et al., 1985). There are a few papers linking a related mouse retrovirus (Mouse mammary tumor virus [MMTV]) as well as other viruses to human breast cancer and other diseases (Lawson and Glenn, 2017). MMTV has convincingly been shown to infect and replicate in human cells (Indik et al., 2007), and even sites of integration of the MMTV provirus in infected human cells have been analyzed (Faschinger et al., 2008).

The exact route of BLV transmission for humans has yet to be discovered; some hypotheses about the BLV infection routes for humans are consuming raw milk and undercooked meat, close contact between humans and bovines, production of vaccines from cell cultures that use bovine fetal serum, and exposition to contaminated meat from butchers and slaughterhouses. However, all these forms of transmission are yet to be confirmed (Schwingel et al., 2019).

Regardless of the lack of conclusive evidence about the role of BLV on human health, this study aims to review the BLV detection frequency in human biological samples to explore the available knowledge about this oncogenic retrovirus that affects the health of cattle herds could affect human health and potentially become a public health problem.

2. Methodology

2.1. Study design

Firstly, we systematically reviewed the literature based on the PRISMA 2020 (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) guidelines. Then we performed a statistical synthesis of the data.

2.2. Identification

We searched the information in the following databases: PubMed, Science Direct, Scielo, Scopus, Google Scholar (Including grey literature), and LILACS. The Mesh-indexed keywords, including "Leukemia Virus, Bovine", "prevalence", "risk factors", and "humans", and their synonyms in Spanish and Portuguese. The keywords were validated in the Thesauri Descriptors in Health Sciences (DeCS) and Medical Subject Headings. The combination gave rise to 9 different search strategies in the six databases. Additional related papers were collected by reviewing

Table 1
Information summary about the 33 articles analyzed.

# I. D.	Title	Publication information Year	Journal	Site*	Refs.
1	Seroepidemiologic testing in men for evidence of antibodies to the feline leukemia virus and bovine leukemia virus	1976	Bibliotheca Haematologica	USA	Caldwell et al. (1976)
2	Seroepidemiologic studies on the possible relationships of human and bovine leukemia: Brief communication	1977	Journal of the National Cancer Institute	USA	Donham et al. (1977)
3	No Involvement of Bovine Leukemia Virus in Childhood Acute Lymphoblastic Leukemia and Non-Hodgkin's Lymphoma	1988	Cancer Research	USA	Bender et al. (1988)
4	Lack of evidence for infection with known human and animal retroviruses in patients with chronic fatigue syndrome	1994	Clinical Infectious Diseases	USA	Heneine et al. (1994)
5	Evaluation of HIV type 1 western blot-indeterminate blood donors for the presence of human or bovine retroviruses	1995	AIDS Res Hum Retroviruses	USA	Sherman et al. (1995)
6	Humans have antibodies reactive with Bovine leukemia virus	2003	AIDS Res Hum Retroviruses	USA	Buehring et al. (2003)
7	Investigation of the bovine leukemia virus proviral DNA in human leukemias and lung cancers in Korea	2005	Journal of Korean Medical Science	South Korea	Lee et al. (2005)
8	Estudio del potencial zoonotico del virus de la leucosis bovina y su presencia en casos de cancer de seno	2006	Universitas Scientiarum	Colombia	Uribe et al. (2006)
9	Serological and genomic detection of bovine leukemia virus in human and cattle samples	2010	Iranian Journal of Veterinary Medicine	Iran	غ et al. (2010)
10	Serological detection of bovine leukemia virus in slaughterhouse workers from San Nicols de los Garza, Nuevo Len, Mexico	2013	African Journal of Microbiology Research	Mexico	Zamora-Avila et al. (2013)
11	Bovine leukemia virus gene segment detected in human breast tissue	2013	Scientific research	Colombia	Giovanna et al. (2013)
12	Bovine leukemia virus DNA in human breast tissue	2014	Emerg Infect Dis	USA	Buehring et al. (2014)
13	Exposure to Bovine Leukemia Virus Is Associated with Breast Cancer: A Case-Control Study	2015	PLoS One	USA	Buehring et al. (2015)
14	Whole genome sequencing of 51 breast cancers reveals that tumors are devoid of bovine leukemia virus DNA	2016	Retrovirology	Mexico / USA	Gillet and Willems (2016)
15	Multiple oncogenic viruses are present in human breast tissues before development of virus associated breast cancer	2017	Infectious Agents and Cancer	Australia	Lawson and Glenn (2017)
16	Bovine leukemia virus linked to breast cancer in Australian women and identified before breast cancer development	2017	PLoS One	Australia	Buehring et al. (2017)
17	Bovine leukemia virus linked to breast cancer but not coinfection with human papillomavirus: Case-control study of women in Texas	2018	Cancer	USA	Baltzell et al. (2018)
18	Bovine leukemia virus presence in breast tissue of Argentinian females and its association with cell proliferation and prognosis markers	2018	Multidisciplinary Cancer Investigation	Argentina	Lendez et al. (2018)
19	Molecular Detection of Bovine Leukemia Virus (BLV) in Patients with Breast Cancer in Khartoum State, Sudan	2019	Scholarena Journal of Cancer Science	Sudan	Ahmed et al. (2020)
20	Presencia de anticuerpos contra el Virus de la Leucosis Bovina (VLB) en mujeres colombianas	2019	Undergraduate dissertation	Colombia	Trujillo Piñeros (2019)
21	Bovine leukemia virus detected in the breast tissue and blood of Iranian women	2019	Microbial Pathogenesis	Iran	Khalilian et al. (2019)
22	Bovine leukemia virus DNA associated with breast cancer in women from South Brazil	2019	Scientific Reports	Brazil	Schwingel et al. (2019)
23	Bovine leukemia virus discovered in human blood	2019	BMC infectious diseases	USA	Buehring et al. (2019)
24	Detecção de DNA do vírus da leucose bovina (BLV) em tecidos mamários humanos	2020	Master's Thesis	Brazil	Nogueira APMdS (2020)
25	High positivity values for bovine leukemia virus in human breast cancer cases from Minas Gerais, Brazil	2020	PLoS One	Brazil	Delarmelina et al. (2020)
26	Absence of bovine leukemia virus in the buffy coats of breast cancer cases from Alabama, USA	2021	Microbial Pathogenesis	USA	Adekanmbi et al. (2021)
27	Prevalence of bovine leukosis virus in water buffaloes in West-central Colombia	2021	Revista Mexicana De Ciencias Pecuarias	Colombia	Flórez et al. (2021)
28	Risk factor for breast cancer development under exposure to bovine leukemia virus in Colombian women: A case-control study	2021	PLoS One	Colombia	Olaya-Galan et al. (2021)
29	Virus de la leucosis bovina (VLB) y evidencias de su potencial zoonótico	2021	Doctoral thesis	Colombia	Olaya-Galán (2021)
30	Virus de la leucosis bovina: evaluación de la presencia del provirus en sangre humana y analisis in silico de los receptores celulares involucrados en su interacción	2021	Master's Thesis	Colombia	Velandia Álvarez (2021)
31	Bovine leukemia viral DNA found on human breast tissue is genetically related to the cattle virus	2021	One Health	Brazil	Canova et al. (2021)
32	Co-Circulation of Bovine Leukemia Virus Haplotypes among Humans, Animals, and Food Products: New Insights of Its Zoonotic Potential	2021	Int J Environ Res Public Health	Colombia	Corredor-Figueroa et al. (2021)
33	Molecular investigation of possible relationships concerning bovine leukemia virus and breast cancer	2022	Scientific reports	Pakistan	Khan et al. (2022)

* Country of origin for the human population studied.

the references of the selected documents.

Some search strategies were the following: in PubMed (leukemia virus, bovine) AND (prevalence) AND (humans); in Scopus (leukemia virus, bovine) AND (risk factors) AND (humans); in LILACS (leukemia Virus, Bovine) AND (epidemiology) AND (humans). The temporality was established according to the first article published, which was found in the 70 s, until November 2022. The database obtained was imported into EndNote 20 for the elimination of duplicates.

2.3. Screening

Once the duplicates were eliminated, the generated database was imported into Rayyan—a web and mobile app for systematic reviews. Studies with the search terms in the title, abstract and keywords, published in English, Spanish, and Portuguese, available in full text, original, in humans, and that estimated the frequency of BLV or related risk factors were included. All remaining articles were excluded. The full texts of the preselected papers, including the bibliography, were reviewed for articles not retrieved during the search in the identification

phase, and disagreements between the two reviewers were resolved.

The following criteria were applied for the selection of qualified studies in this research:

(a) Original articles that estimate the frequency of BLV in humans or the probable risk factors associated with its detection in humans.
(b) Studies that specify the technique used for the detection of BLV.

While those studies focused on the detection and risk factors associated with BLV transmission in animal species were excluded.

2.4. Inclusion

the papers that met the criteria described above were included for the qualitative synthesis of the variables title, authors, year, place of production and publication, study population, identification method, target and sample used, number of people evaluated, and number of positive people. The data obtained were recorded in a spreadsheet format (Microsoft Excel) table.

The process of identification, screening and analysis of the files was carried out by two researchers independently following the PRISMA 2020 statement checklist. The methodological quality was evaluated with the criteria of the STROBE guide (Strengthening the Reporting of Observational Studies in Epidemiology). Though this is an editorial guide, it contains standards that allow evaluation of the methodological quality of the descriptive studies.

2.5. Analysis

Data tables summarize the information extracted from the papers qualitatively, registering for each one the variables title, author(s), year of publication, year of development of the study, journal, URL, country of publication, country of development of the study, language, type of study, sample, study population, the technique and target of diagnosis, number of people studied, number of positive people and reported prevalence. To consolidate the information reported in the studies identified from the systematic review (n = 33), a meta-analysis was performed to estimate the global proportion of positives. Statistical synthesis was carried out to assess the global frequency of BLV (data available from 31 studies) and the Odds Ratio with 95% confidence intervals (data available from 12 studies) (Fig. 1). Two global measures were estimated, positivity frequency and log odds of positivity, considering people with cancer as exposed. The included studies presented a lot of variabilities I^2 >90%, so the global effect was estimated with a random-effects model. We performed the statistical analysis using Jamovi version 2.3.18.

3. Results

In the initial search, we identified 759 articles by combining the keywords in 9 search strategies in the six databases, PubMed (n = 135), Science Direct (n = 38), Scielo (n = 1), Scopus (n = 113), Google Scholar (n = 187) and LILACS (n = 285). Of these, we removed 241 papers because they were duplicating. Then, two researchers independently read and analyzed the title, keywords, and abstracts of 518 articles. Reports that focused on general aspects of the virus were research review articles or written in a language other than English, Spanish, or Portuguese were also excluded. We selected 43 papers that met the inclusion criteria for reading the full text. Of these, 18 articles that detected the presence of the virus or reported risk factors associated with the virus in animal samples or farm products were excluded resulting in 25 preselected articles. In addition, eight papers identified by screening the bibliography after reading the full text of the 25 preselected articles were included in the present study. Finally, in this study, we analyzed 33 papers (Table 1).

The selected scientific articles were published mainly in the United States (n = 13), the United Kingdom (n = 5), and Colombia (n = 4) and were published between the years 1976 and 2022. The type of studies consisted mainly of descriptive studies (67%) and cases and controls (33%) (Table 2). In total, in the 33 papers evaluated, samples from 10,398 humans were analyzed, of which about 63% were women. Analyzed studies were carried out in countries such as Pakistan (n = 2790; 26.8%), the USA (n = 2665; 25.6%), Brazil (n = 1902; 18.3%), Colombia (n = 1052; 10.1%), Iran (n = 931; 9%), South Korea (n = 679; 6.5%), Australia (n = 140; 1.3%), Argentina (n = 89; 0.9%), Sudan (n = 52; 0.5%), Mexico (n = 28; 0.3%), and there were 70 participants from no specified origin (0.7%) (Fig. 2).

Table 2
Frequency of different types of variables from the 33 studies included in this systematic review.

Variable	n	%
Study		
Descriptive	22	67
Case-control	11	33
Population group		
Female	6595	63
Male	318	3
Kids	293	3
Not specified	3192	31
Sample		
B.M.	1	2.6
BM & PB	1	2.6
BTFFP	19	50
Fresh breast tissue	1	2.6
P.B./ Cell fraction	8	21
P.B./ Serum	5	13.2
P.B./ Cell fraction & serum	3	8
Technique		
AGID assay	3	6.8
Complement fixation test	1	2.3
ELISA	4	9
IHC	3	6.8
Immunoblotting	1	2.3
Immunoperoxidase	1	2.3
Nested PCR	13	29.5
Nested PCR & Nested In situ PCR	1	2.3
PCR	6	13.6
PCR In situ	7	15.9
qPCR	1	2.3
Southern blot	1	2.3
WSG	2	4.5
Target		
env	8	13
gag	12	19
tax	16	26
pol	5	8
px	1	2
LTR	4	6
WSG	1	2
gp51	4	6
p24	6	10
Not specified	5	8

P.B.: Peripherally blood, BTFFP: Breast tissue specimen fixed in neutral formalin and embedded in paraffin, B.M.: Bone marrow, WSG: Whole genome sequencing, IHC: Immunohistochemistry, AGID: Agar gel immunodiffusion, ELISA: Enzyme-Linked ImmunoSorbent Assay, PCR: Polymerase Chain Reaction.

Human samples analyzed for BLV presence were peripheral blood (42%), neutral formalin-fixed paraffin-embedded breast tissue specimen (50%), fresh breast tissue (2.6%), and others. One study analyzed two samples from the same person, seeking to correlate the results of BLV detection in peripheral blood and fresh breast tissue. The authors found that BLV was detected both in blood and breast tissues with a correlation of 94% in the positive samples of the study (Olaya-Galan et al., 2021; Corredor-Figueroa et al., 2021) (Table 3).

Serological studies based on the detection of antibodies against BLV in humans show conflicting results. Studies based only on classical serological techniques such as complement fixation and agar gel

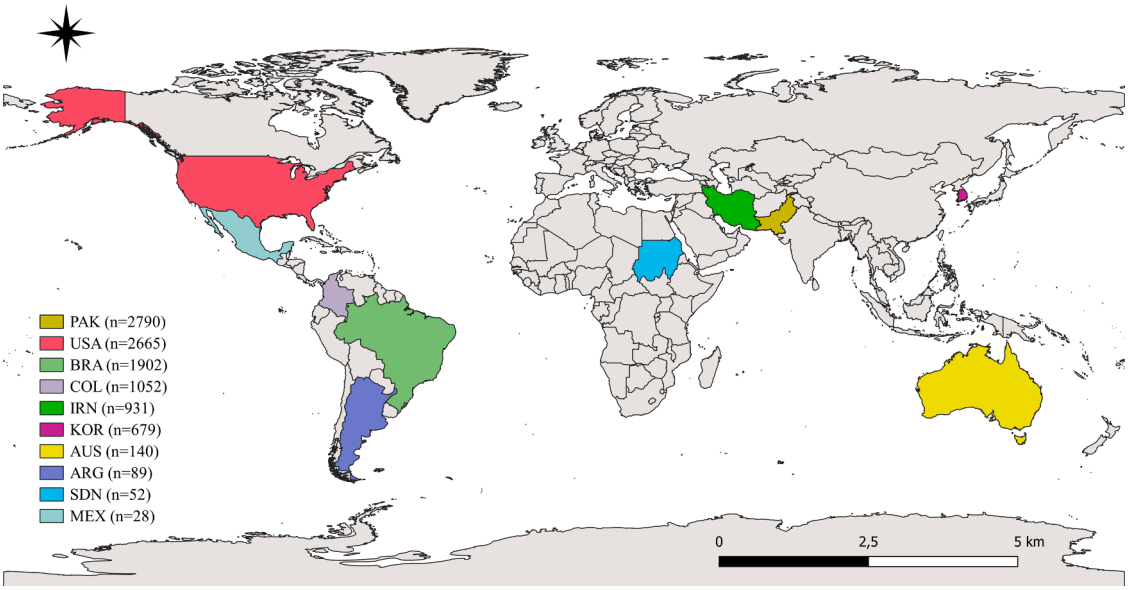

Fig. 2. Geographic location of the origin of the populations included in the 33 articles analyzed in this study. PAK: Pakistan; USA: United States of America; BRA: Brazil; COL: Colombia; IRN: Iran; KOR: South Korea; AUS: Australia; ARG: Argentina; SDN: Sudan; MEX: Mexico, n: Number of people analyzed by country.

immunodiffusion (AGID), showed no evidence of human antibodies against BLV (Caldwell et al., 1976; Donham et al., 1977). One study compared the sensitivity of AGID test versus the immunoblot, which is a more sensitive technique. It detected anti-BLV antibodies by immunoblot in 39% of serum tested negative by AGID (Buehring et al., 2003). In a study which evaluated the presence of BLV by AGID and PCR in the same blood sample, they found a positivity of 7% with the AGID technique and 0% with the PCR test, for which the authors conclude that the presence of antibodies may be due to previous exposure to virus antigens and does not guarantee an active viral infection (Zamora-Avila et al., 2013). On the other hand, solid phase serological techniques, such as enzyme immunoassay (ELISA) using the virus p24 antigen, showed that humans have IgG, IgM and IgA antibodies against BLV, indicating that antibodies reactive with the BLV antigen capsid may serve as a biomarker for BLV exposure (Buehring et al., 2019; غن et al., 2010).

BLV p24 protein was detected in human breast tissue samples analyzed by Immunohistochemistry (IHC), indicating the presence of viral proteins as evidence of active viral replication (Buehring et al., 2014; Buehring et al., 2015).

The samples were mainly analyzed by molecular techniques such as nested PCR (Polymerase Chain Reaction) using different target genes such as *tax, gag, env, pol*, and LTR. One study analyzed the whole genome of the BLV. The other studies used molecular and serological virus detection techniques (Table 3).

In general, the results of PCR techniques indicate the presence of the virus in the proviral stage (integrated into the host cell genome). Molecular techniques employed include standard PCR, which is based on DNA extracts from target tissues and cannot be used to identify the location of viral gene sequences in specific cell types, unlike in situ PCR which can identify viral gene sequences in particular cells, studies using in situ techniques were able to confirm that BLV was located within mammary epithelial cells (Buehring et al., 2014; Lendez et al., 2018; Buehring et al., 2017; Buehring et al., 2015; Lawson and Glenn, 2017; Olaya-Galan et al., 2021).

Only one study evaluated the presence of BLV using probe-based qPCR, which is a much more sensitive and specific molecular technique and is widely used for the evaluation of gene expression of specific genes in a relative way. However, neither in this study nor in any of the studies included in this review was gene expression assessed. The authors did not detect the presence of BLV by qPCR or WSG in peripheral blood samples from women with cancer; however, some important aspects in the methodology of this study are not reported, such as the negative controls or the inhibition controls they used to confirm the functionality of their detection assay (Adekanmbi et al., 2021).

Two studies performed massive genome sequencing to identify specific viral gene segments but did not yield positive results (Gillet and Willems, 2016; Adekanmbi et al., 2021). This result may be because whole genome sequencing detects only viral DNA integrated into the human genome, which may be present at too low concentrations (<1% of reads) to be caught without amplification (Buehring et al., 2019).

Nested PCR is a modification of the standard PCR that was designed to improve sensitivity and specificity. Due to this, it was the most used technique in the different studies included in this review (29.5%), reporting different percentages of positivity (Table 3). A possible explanation for the negative results could be due to low viral loads, partial deletion or polymorphism of the target viral gene that could hinder hybridization with viral DNA, accidental loss of virus during cell division which can result in a virally transformed cell with no detectable virus ("hit-and-run virus" phenomenon) (Joshi and Buehring, 2012).

The results of the studies are very heterogeneous with the index I^2 =99%, so the global effect was estimated with a random-effects model. Overall, we observed high heterogeneity in the virus detection frequency according to the sample and the technique involved. The analysis was carried out separately according to the design of the studies. In the descriptive ones, a slightly lower frequency was found at 24% (12% to 36%), while in the case-control studies was 34% (16% to 52%). We observed that the frequency of positivity ranged from 0 to 87%. For this reason, the overall estimate resulted in a positivity frequency of 27% with an interval between 17 and 37% (Fig. 3).

Only the study from Corredor-Figueroa et al. (Corredor-Figueroa et al., 2021) analyzed the potential exposure factors related to the presence of the virus. Through bivariate analysis, they found a correlation between BLV presence and the consumption of dairy products, such as raw milk and homemade yoghurt, and the number of dairy products consumed by the person. Also, they observed a significant statical relationship between virus presence and age (≥50, <50; p = 0.039) and the city of origin of the participants (Bogotá or other, p = 0.036). This study estimated that women with higher consumption of raw milk and homemade yoghurt had a higher risk of the presence of BLV (OR = 2.424, 95% CI: 1.063–5.527, p = 0.035).

Fifteen studies reported Bovine Leukemia Virus gene sequences by PCR in tissue samples from human breast cancers (Table 3). In a PCR-

Table 3
Frequency of BLV detection by sample type, population analyzed, and technique used.

# I. D.	Sample	Population	Population characteristics	Technique	n	Frequency of positive samples
1	P.B./ Serum	Volunteer men and women	Volunteer men and women	Complement fixation test	192	0%
2	P.B./ Serum	Volunteer men and women	Leukemia patients	AGID assay	358	0%
			Veterinarians			
			Dairy farmers			
			Control group			
3	B.M. and P.B./ Serum	Kids with ALL or NHL	Cases	Southern blot	293	0%
		Healthy kids	Control group			
4	P.B./ Cell fraction	Chronic fatigue syndrome case-patient	Health control	PCR	42	0%
			Chronic fatigue syndrome case-patient			
5	P.B./ Cell fraction	Blood donors	Blood donors	PCR	20	0%
6	P.B./ Serum	Volunteer men and women	Volunteer men and women	Immunoblotting	257	74.3%
				AGID assay	25	0%
7	B.M.	Leukemia patients	Acute myeloid leukemia patients	Nested PCR	517	0%
			Chronic myelogenous leukemia cases patients			
	BTFFP	Lung cancer cases	Lung cancer cases		162	
8	BTFFP	Women with breast surgery	Malignant breast cancer	Immunoperoxidase	56	7%
9	P.B./ Cell fraction & serum	Volunteer men and women	Volunteer men and women	Nested PCR	77	9%
				ELISA	454	13%
10	P.B./ Cell fraction & serum	Slaughterhouse workers	Slaughterhouse workers	AGID assay	28	7%
				PCR		0%
11	BTFFP	Women with breast surgery	Benign (controls)	PCR	106	45%
			Malignant (cases)			35%
12	BTFFP	Women with breast surgery	Benign and malignant breast surgery	Nested PCR/ Nested-*In situ* PCR	219	44%
				IHC	215	16%
13	BTFFP	Women with breast surgery	Benign (controls)	PCR *in situ*	239	29%
			Malignant (cases)			59%
			Premalignant			38%
			NA	IHC	236	5%
14	BTFFP	Women with breast surgery	DNA sequences from whole genomes of normal breast tissues	WSG	70	0%
			DNA sequences from whole genomes of breast tumors			
15	BTFFP	Women with breast surgery	Benign (controls)	PCR *in situ*	44	35%
			Malignant (cases)			74%
16	BTFFP	Women with breast surgery	Benign (controls)	PCR *in situ*	96	41%
			Malignant (cases)			80%
17	BTFFP	Women with breast surgery	Benign (controls)	PCR *in situ*	216	20%
			Malignant (cases)			57%
			Premalignant			34%
18	BTFFP	Women with breast surgery	Benign (controls)	PCR *in situ*	89	25%
			Malignant (cases)			22.4%
19	BTFFP	Women with breast surgery	Breast cancer patients	PCR	52	3.8%
20	P.B./ Serum	Volunteer women	Volunteer women	ELISA	226	0%
21	BTFFP	Women with breast surgery	Malignant (cases)	Nested PCR	200	24%
			Non-malign			2%
			Non-tum			4%
			Malignant (cases)			6%
			Non-malign			0%
			Non-tum			2%
	P.B./ Cell fraction	Volunteer women	Volunteer women		200	17%
22	BTFFP	Women with breast surgery	Benign (controls)	Nested PCR	144	14%
			Malignant (cases)			31%
	P.B./ Serum	Blood donors	Blood donors	ELISA	1500	0.13%
23	P.B./ Cell fraction & serum	Volunteer women	Volunteer women	Nested PCR	95	38%
				ELISA		58%
24	BTFFP	Women with breast surgery	Benign (controls)	Nested PCR	111	73%
			Malignant (cases)			81%
			No diagnosis			45%
25	BTFFP	Women with breast surgery	Benign (controls)	Nested PCR	88	59%
			Malignant (cases)			96%
26	P.B./ Cell fraction	Volunteer women	Breast cancer patients	qPCR	258	0%
				WSG		
27	P.B./ Cell fraction	Volunteer men and women	Volunteer men and women	Nested PCR	9	0%
28	P.B./ Cell fraction	Women with breast surgery	Benign (controls)	Nested PCR/PCR *in situ*	158	48%
			Malignant (cases)			62%
	Fresh breast tissue		Benign (controls)	IHC		10%
			Malignant (cases)			12%
29	BTFFP	Dead women	Benign	Nested PCR	145	71%
				PCR *in situ*		
30	P.B./ Cell fraction	Volunteer men and women	Volunteer men and women	Nested PCR	352	27.3%
31	BTFFP	Women with breast surgery	Women with breast surgery	PCR	59	86%
				Nested PCR		

(*continued on next page*)

Table 3 (*continued*)

# I. D.	Sample	Population	Population characteristics	Technique	n	Frequency of positive samples
32	P.B./ Cell fraction BTFFP	Women with breast surgery	Same population and technique that study #ID 28			
33	BTFFP	Women with breast surgery	Benign (controls) Malignant (cases)	Nested PCR	2790	10% 27%

ID: Corresponds to the same article in Table 1, ALL: Acute Lymphoblastic Leukemia, NHL: Non-Hodgkin's Lymphoma, P.B.: Peripherally blood, BTFFP: Breast tissue specimen fixed in neutral formalin and embedded in paraffin, B.M.: Bone marrow, WSG: Whole genome sequencing, AGID assay: Agar gel immunodiffusion assay, ELISA: Enzyme-Linked Immunosorbent Assay, PCR: Polymerase Chain Reaction, IHC: Immunohistochemistry.

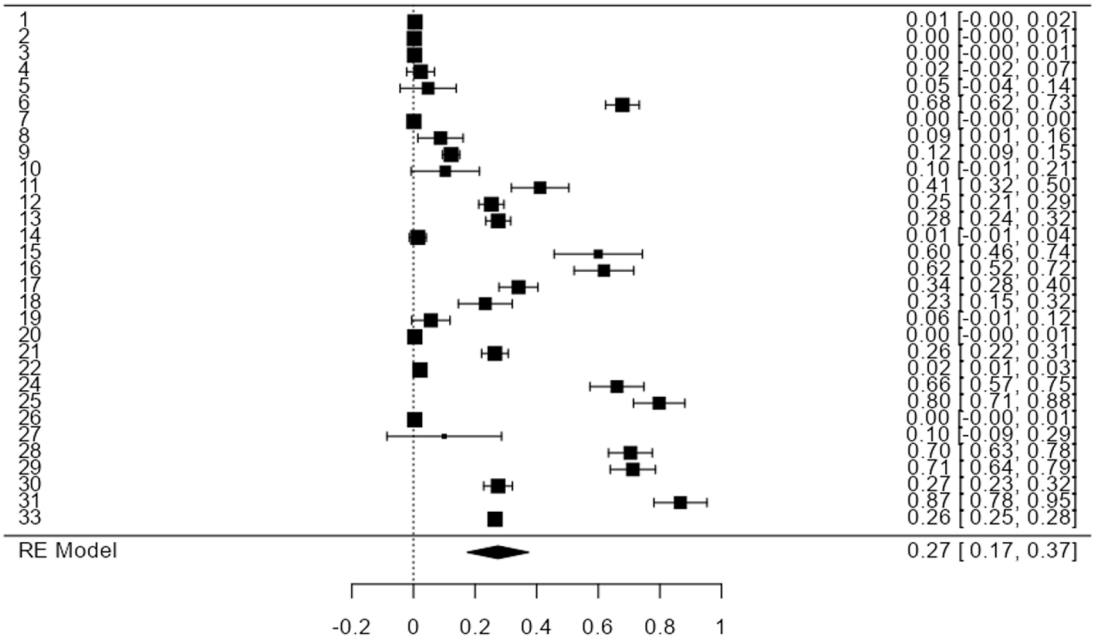

Fig. 3. Forest plot of the individual and global BLV frequencies of positivity detected in the 31 analyzed studies. It shows the estimates of each study, where studies with positivity from 1% to 87% are observed; in eight studies, there were percentages of positivity greater than 50%, eight presented zero positive cases, and 15 studies had positivity between 2 and 41%; the overall estimate of positivity was 27% with a range between 17 and 37%. An analysis excluding data from the four gray literature documents (Trujillo Piñeros, 2019; Nogueira APMdS, 2020; Olaya-Galán, 2021; Velandia Álvarez, 2021) showed the BLV frequencies of positivity decreased by 2%, from 27% to 25%, and the confidence interval ranged from 14% to 35%.

based case-control study, 67 (59%) of 114 US breast cancers tested positive for BLV compared with 30 (29%) of 104 normal breast controls: odds ratio 3 (Buehring et al., 2015). A similar prevalence pattern is observed in the studies included in this review, which had been designed as cases and controls ($n = 12$) (Table 3). In another study carried out in the USA, an increase in the prevalence of BLV was observed in benign breast tissues (20%), premalignant breast tissues (34%) and malignant breast tissues (57.4%) (Baltzell et al., 2018).

In the papers we reviewed, BLV's prevalence is significantly higher in breast cancer tissues compared to normal or benign control breast tissues. The presence of the infectious agent in normal or benign tissues before cancer development is a key criterion for a causal role in cancer (Hill, 1965). A study using the PCR-in situ technique identified BLV in 23 (74%) of 31 benign breast biopsy tissues 3 to 10 years before the development of BLV-positive breast cancers in the same Australian patients (Buehring et al., 2017).

In 12 of the studies, they compared positivity between women with and without breast cancer, for which we evaluated the effect by estimating the log odds and the odds ratio (OR) was assessed. Two studies found a negative point estimate, that is, it decreases the possibility of being positive; the other ten studies showed results indicating that having cancer increases the probability of being positive, and nine showed statistically significant values (intervals do not include zero). The overall estimate was 1.06, which means that the presence of cancer increases the possibility of being positive. The chance of being positive is 2.88 times compared to those without breast cancer, which can vary between 1.84 times and 4.53 (Fig. 4).

4. Discussion

This review included 10,398 people evaluated using different techniques to detect the presence of BLV. To date, the frequency of detection of BLV by analysing sequences, antigens, or antibodies have been found in 27% (Ranging between 17 and 37%) of people evaluated. The BLV frequencies in the population-assessed groups presented a significant heterogeneity according to the characteristics of the population, the sample and the diagnostic technique used. For example, in a women group diagnosed with a malignancy in the breast tissue, the presence of the virus was evaluated using the nested-PCR method and found a positivity of 95.9%, in contrast in the same study, in the group of women without a diagnosis of malignancy in the breast tissue, the BLV frequency was 59% (Delarmelina et al., 2020).

In general, molecular biology techniques have high sensitivity and specificity. For example, the in-situ PCR technique decreases cross-contamination with the positive control or between samples because DNA is not extracted and fixed within control cells and human tissues and cannot escape and generate cross-contamination. Any contaminating molecule amplified during PCR is eliminated during the rinse

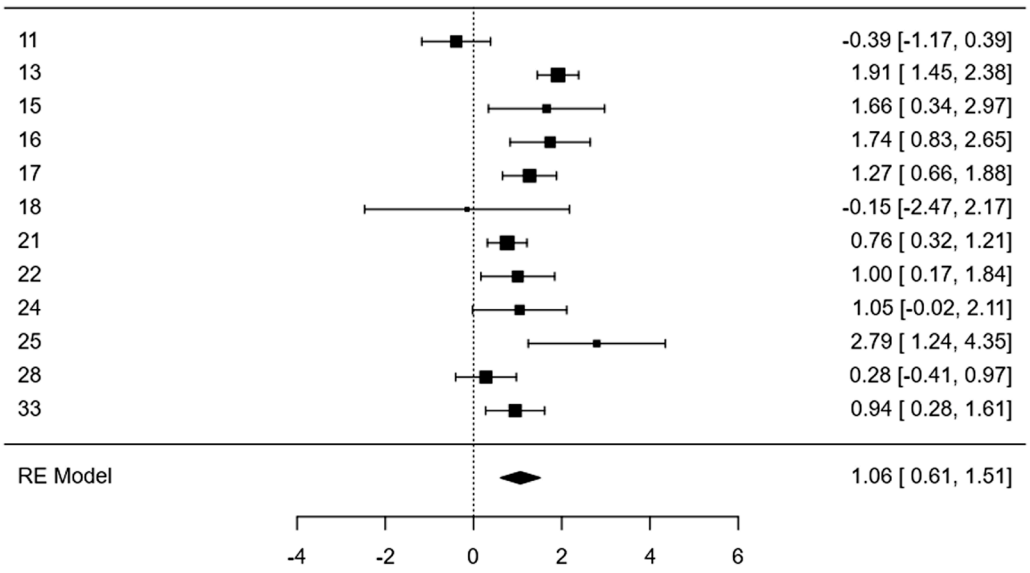

Fig. 4. Log of Odds of the Individual and Global data from 12 analyzed studies. The figure shows how cancer increases the possibility of being positive for BLV in most studies (10/12). Overall, the log odds estimate showed that having cancer increases the chance of being positive for the virus, OR global=e^1.06=2.88 (1.84–4.53), which means that in women with cancer, the possibility of being positive for BLV is 2.88 times more than those without cancer, varying between 1.84 and 4.53 times.

steps, while the amplified intracellular DNA remains fixed within the cellular architecture (Baltzell et al., 2018).

In the study in which AGID-positive and PCR-negative results were obtained, the authors conclude that one of the following conditions is likely: (1) Constant exposure may have occurred without infection, eliciting an aroused immune response; however, the virus could not integrate into the human genome. (2) Infected lymphocytes may have become trapped in lymphoid organs and were not in the peripheral circulation. (3) The number of provirus particles may have been deficient, and nested PCR would have been needed to increase sensitivity (Zamora-Avila et al., 2013).

The most common molecular techniques used to detect BLV presence were directed at different targets of the proviral DNA, such as *env, gag, pol, tax,* and LTR. Most studies use only one of these targets, and some studies use two or more of these, which may increase the likelihood of detecting the virus since partial genome deletions after integration into host cells are considered an essential mechanism of immune response evasion (Rosewick et al., 2017). In a study of the HTLV-1 virus carried out by Kamihira et al., which is closely related to BLV, deletions of the provirus integrated into the host genome began in the *gag* region, followed by deletions of the *pol* and *env* genes; in contrast, *tax* and LTR regions were the least frequently deleted genes (Kamihira et al., 2005). Analogous results were observed in specimens from cattle infected with BLV; deletions involving parts of *gag* and *env* and all of the *pol* were frequent (Gillet et al., 2007). Therefore, choosing hybridization primers or probes is an important point to consider in designing PCR assays and could be another potential reason for negative results.

Using whole genome sequencing methods, BLV was not identified in 70 breast tissue samples or 20 peripheral blood samples from women with breast cancer (Gillet and Willems, 2016; Adekanmbi et al., 2021). The reason for the negative result based on whole genome sequencing is unclear. One plausible explanation is that complete genome sequencing techniques are not as sensitive as amplification techniques such as PCR, and the authors assumed that BLV would have to be present in all cells in the tissue, which may not be the case. Retroviral genomes rarely exceed 10–12 kb and constitute a minor fraction of the infected host cell genome. The infected cell type may include only a small fraction of the sample, and infected cells may contain a relatively low number of copies of the viral genome (Lawson and Glenn, 2017).

Early studies of BLV in humans based on serologic tests such as AGID and complement fixation tests reported no evidence of the virus in human samples. A possible explanation for some of the negative results could be that sufficiently sensitive reagents and techniques (Buehring et al., 2003). Serological methods based on detecting antibodies directed to virus antigens such as p24 and gp51. This last is an envelope glycoprotein subject to variation within different viral subspecies. Thus, the epitope targeted by the detecting monoclonal antibody could not be present on BLV sub-species that circulate in the region studied and, as such, infected individual's bovine or human (Schwingel et al., 2019). The detection of antibodies against viruses was widely used in different studies; these techniques are useful for diagnosing viral diseases; however, in the case of BLV infections, relying on antibodies to prove the infection has several disadvantages. BLV may not express the p24 capsid protein in blood cells and may not replicate there (Buehring et al., 2019). Studies in cattle indicate that lymphocytes harbouring the BLV provirus rarely produce extracellular virions or express viral proteins, even though cattle have antibodies to BLV (Buehring et al., 2003)

None of the included studies was specifically designed to determine the route of exposure of humans to BLV. Some of the articles mention the consumption of raw milk as a potential transmission route, but this was not a variable under study. BLV transmission from cattle to humans is also plausible, as BLV is widespread in beef and dairy herds. Although pasteurization renders the virus noninfectious, and presumably thorough beef cooking does as well, many people have drunk raw milk and eaten raw or undercooked meat at some point (Buehring et al., 2015).

In the studies included in this review, we observed that the prevalence of BLV is significantly higher in breast cancer tissues compared to normal or benign control breast tissues. In 12 of the studies, they compared positivity between women with and without breast cancer, for which we evaluated the effect by estimating the log odds and the odds ratio (OR) was calculated. The overall estimate was 1.06, which means that the presence of cancer increases the possibility of being positive. The possibility of being positive is 2.88 times compared to those without breast cancer, which can vary between 1.84 times and 4.53. This observation supports that this oncovirus may have a potential role in human breast cancer.

Although many steps are needed to establish causality for any disease, a statistically significant association between the disease and the suspected agent is the first step. Validation of this association by other investigators in different populations is an essential subsequent step

(Hill, 1965). Although case-control studies are not conclusive in themselves, this type of study is widely accepted as a valid method of establishing an association between the presence of a causative agent and the outcome of the disease. It would be desirable for prospective studies that show that the viral infection preceded the development of detectable cancer to support the idea of a causal association of BLV with breast cancer. In addition, it is crucial to carry out studies focused on demonstrating that the virus can transform normal cells into malignant cells, understand the oncogenic mechanism of the virus and identify the route of viral transmission.

Depending on the country of origin, all studies have variable percentages of BLV presence, possibly due to different lifestyles, diets, ages, types, and the number of sample sizes used, and techniques applied to detect the virus occurrence. We observed high heterogeneity in the data of frequencies of BLV detection, estimating an overall frequency of 27% (Ranged between 17 and 37%). The percentage of BLV detected in human samples by any study suggests the need to investigate further its transmission route to humans and the role of the virus in developing and progressing diseases (Khalilian et al., 2019).

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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.

Data availability

Data will be made available on request.

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