Journal of Clinical and Translational Research

ACCSCIENCE PUBLISHING
Volume 10 Issue 3, June 2024
ISSN: 2424-810X (online) ISSN: 2382-6533 (print)

ABOUT JCTR

Aims and scope

The Journal of Clinical and Translational Research (JCTR) is an open access, peer-reviewed, multidisciplinary scientific journal that publishes studies with at least an ex vivo, in vivo, or clinical component. The published research is centered on any clearly defined clinical problem, which may comprise a disease or the basis of disease, a form of therapy or intervention, and clinical diagnostics or prognostics. Articles (original research, reviews, technical reports, medical hypotheses, commissioned articles, special issue articles, and editorials) are published continuously online and bimonthly in print. Studies performed in cells only will generally not be accepted unless they contain critical data that are in line with the scope of the journal. Some examples of such studies include molecular pathways that lie at the basis of a disease, novel biotechnological approaches for e.g., the production of drugs, or new techniques that improve clinical diagnostics and prognostics. Articles that combine preclinical and clinical data are given priority. Contributions from academic institutions and industry are welcome.

The research areas that JCTR covers include but are not limited to:

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Intensive care medicine
Ophthalmology
Neurology and neurosciences
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Pathology
Clinical physics
Epidemiology
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Nephrology
Dermatology
Endocrinology and metabolism
Anesthesiology
Radiology and nuclear medicine
Clinical chemistry
Genetics and epigenetics
Global health
Nutrition
Immunology
Virology
Biomedical engineering

Key features

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- No word count or reference restrictions
- Double blind review process to minimize bias
- Rapid online publication of articles upon acceptance
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Volume 10 • Issue 3 • June 2024
ISSN 2382-6533 (print) ISSN 2424-810X (online)

JOURNAL OF CLINICAL AND TRANSLATIONAL RESEARCH

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Journal of Clinical and Translational Research

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Journal of Clinical and Translational Research

Journal homepage: http://www.jctres.com/en/home

ORIGINAL ARTICLE

Alterations of biliary and gut microbiota in relation to gallstone formation

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ARTICLE INFO

Article history:
Received: 04 October 2023
Accepted: 22 January 2024
Published Online: May 28, 2024

Keywords:
Gallstones
Gut microbiota
Biliary microbiota
Diversity
Microflora structure

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ABSTRACT

Background: The gut and biliary microbiota are important components of the complex microecology system in the human body. However, it is often difficult to obtain bile in clinical practice to manage gallstone diseases, warranting further microbiota research to evaluate the relationship between biliary microbiota and gallstone formation.
Aims: We aimed to characterize the diversity and alterations of biliary and gut microbiota in patients with gallstones and analyze their possible correlations to gallstone formation.
Methods: We collected gallstones, bile, gallbladder mucosa, and feces from 21 patients with gallstone disease during operation and fecal samples from 20 healthy subjects without gallstones. We performed high-throughput sequencing of the V3-V4 regions of the 16S rRNA gene in the gallstone and control groups and analyzed the final optimization sequence.
Results: We identified a total of 23,427 operational taxonomic units. *Achromobacter* ($P = 0.010$), *Faecalibacterium* ($P = 0.042$), and *Lachnospira* ($P = 0.011$) were significantly reduced, while *Enterococcus* ($P = 0.001$) was increased in the gallstone group. The diversity and composition between the biliary and gut microbiota in gallstone patients had statistical differences. The diversity of gut microbiota was significantly higher than that of biliary microbiota ($P < 0.05$). In addition, linear discriminant analysis (LDA) >4 indicated that the characteristic flora was specific to five samples. *Prevotella* and Proteobacteria had LDA values >4 in the feces and both bile and gallbladder mucosa, respectively, of patients with gallstones.
Conclusion: The biliary and gut microbiota of patients with gallstones displayed bacterial heterogeneity. *Prevotella* and Proteobacteria may serve as biomarkers for dysbacteriosis in patients with gallstones, suggesting that alterations of biliary and gut microbiota are involved in the formation of gallstones. This study highlights the potential application of fecal microbiota transplantation technology in the treatment of gallstone diseases.
Relevance for Patients: Microecology of the digestive tract is closely related to the formation of gallstones, providing new ideas for the prevention and treatment of patients with gallstones.

1. Introduction

Gallstones are crystal deposits in the biliary system, including the gallbladder and bile duct. Gallstones commonly affect 10 – 20% of the global adult population [1], and the resultant cost of gallbladder disease constitutes a major health burden [2]. With changes in dietary patterns and the aging population, the incidence of gallstones is increasing. Approximately 5% of patients with gallstones develop acute cholecystitis, suppurative cholangitis, severe acute pancreatitis, biliary fistula, and other serious complications [3,4]. In addition, gallstones have been associated with an increased risk of chronic diseases, such as diabetes and cardiovascular disease [5,6]. There is compelling evidence

DOI: https://doi.org/10.36922/jctr.23.00118

supporting the idea that long-term gallstone stimulation can lead to the transformation of benign hyperplasia into malignant gallbladder mucosal epithelial cells, leading to gallbladder cancer [7,8]. Recent studies have also reported that patients with asymptomatic gallstones have a significantly increased risk of the right-sided colon cancer after 15 years [9,10]. Therefore, more emphasis should be placed on new prevention strategies against the formation of gallstones.

The current understanding of the pathogenesis of gallstones is very complex, mainly involving local and systemic factors. The local factors include gallbladder wall motility disorder, local persistent immune-mediated inflammation, mucin secretion and accumulation, cholesterol supersaturation, and solid crystal precipitation [11,12]. Likewise, the systemic factors generally include gene polymorphism, epigenetic factors, expression and activity of nuclear receptors, insulin resistance, slow intestinal peristalsis, and increased cholesterol absorption [11,13]. The activity of gut microbiota can dysregulate the lipids in bile and increase the excretion of bile acids, leading to the development of gallstones [14]. Some patients with gallstones suffer from discomforts such as belching, abdominal pain, abdominal distension, and constipation for a long time. In recent years, much emphasis has been placed on understanding whether the formation of gallstones is related to gut microbiota disorders. The screening of biliary and intestinal microbiota has evolved from microscopic characterization and identification to various culture technologies (e.g., bacterial smear and culture methods) and molecular biology techniques. Nonetheless, the bacterial smear method has limitations in identifying bacteria, while the bacterial culture method has a low positive rate and limited efficacy in studying biliary tract microorganisms. Conversely, molecular biology techniques (e.g., polymerase chain reaction [PCR] amplification, fluorescence *in situ* hybridization, gene chip technology, 16S rRNA gene sequencing, and whole genome sequencing) have displayed advantages in accuracy, reliability, and repeatability in studying the diversity and subtle changes of microbiota. Among them, 16S rRNA gene sequencing is the most suitable method for bacterial phylogeny and species classification.

In this study, the V3-V4 variable regions of the 16S rRNA gene were sequenced to reveal the diversity of biliary and gut microbiota in patients with biliary stones. We aimed to investigate the alterations of biliary and gut microbiota and their possible correlation to gallstone formation.

2. Methods

2.1. Study design

We recruited patients who underwent a physical examination at the Beijing Tiantan Hospital affiliated with the Capital Medical University from November 2019 to November 2020 based on the following inclusion and exclusion criteria:

i. Inclusion criterion: Patients with gallstones that were confirmed by abdominal B-mode ultrasound or computed tomography.
ii. Exclusion criteria: Patients who received antibiotics within the past 3 months; the presence of other serious metabolic diseases, such as severe obesity, uncontrollable hyperlipidemia, and diabetes; patients who took a large dose of probiotics 3 months before the study; patients who took somatostatin or other drugs affecting gallstone formation, such as oral biliary acid therapy and proton-pump inhibitors; pregnant women or long-term contraceptive users; patients who underwent endoscopic retrograde cholangiopancreatography or intestinal surgery; and patients who have primary sclerosis cholangitis, primary biliary cholangitis, or Gilbert diseases.

Based on the above selection criteria, we included 21 patients who underwent gallstone surgery (gallstone group) and 20 healthy patients without gallstones (control group). Clinical information on all patients was obtained, including gender, age, body mass index (BMI), and cholesterol level. Gallstone, bile, gallbladder mucosa, and feces specimens were collected from the gallstone group, and feces were collected from the control group. This study was performed in accordance with the ethical standards of the responsible committee on human experimentation (International Council for Medical Sciences) and with the Helsinki Declaration. This study was approved by the Institutional Research Ethics Committee of the Beijing Tiantan Hospital, Capital Medical University (KY 2020-032-02). All experimental personnel involved in specimen collection and transportation received professional training.

2.2. Sample collection and processing

During laparoscopic cholecystectomy, general surgeons collected the gallstones, bile (2 mL), and gallbladder mucosa (about 1 × 1 cm in size). The assistant cleaned the surface of the gallstones and mucosa specimens with sterile normal saline and stored them in sterile and labeled cryopreservation tubes. Fresh feces from the gallstone group were collected before surgery, while feces from the control group were collected after admission. Approximately 5 g of fresh feces were collected and placed in sterile and labeled cryopreservation tubes for bacterial community detection. All specimens from the study participants were placed in liquid nitrogen for at least 3 min for rapid freezing and then stored in a freezer at −80°X.

2.3. DNA extraction and bacterial 16S rRNA amplification and sequencing

DNA was extracted from samples (0.5 g) using the QIAamp PowerFecal DNA Kit (QIAGEN, Germany) according to the manufacturer's protocols. Subsequently, the V3-V4 regions of the bacterial 16S rDNA gene were amplified by PCR using primers 16s-336F (5'-GTACTCCTACGGGAGGCAGCA-3') and 16s-806R (5'-GTGGACTACHVGGGTWTCTAAT-3'). The first PCR reaction was performed in a 25 μL mixture containing 5 μL 5× GC Buffer, 0.5 μL KAPA dNTP Mix, 0.5 μL KAPA HiFi HotStart DNA polymerase (KAPA Biosystems, America), 0.5 μL forward primer (10 pM), 0.5 μL reverse primer (10 pM), and 50 – 100 ng of template DNA. PCR cycling included 95°C denaturation for 3 min, followed by 25 cycles at 95°C for 30 s, 55°C annealing for 30 s, and 72°C elongation

DOI: https://doi.org/10.36922/jctr.23.00118

for 30 s, with a final extension at 72°C for 5 min. The amplified products from gallstones, bile, gallbladder mucosa, and feces samples were verified by gel electrophoresis with a 1.5% agarose gel, a mixture of 3 μL PCR product and 3 μL 3× loading buffer, and 3 μL 100 bp ladder marker (Yingwei Jieji Trading, China) at 100V voltage over 35 – 40 min.

Agencourt AMPure XP (Beijing Huaruikang Technology, China) was used to purify the 16S V3-V4 amplicons to be free of primers and primer-dimer species. The second PCR reaction was performed in a 25 μL mixture containing 5 μL 5× GC buffer, 0.75 μL KAPA dNTP mix, 0.5 μL KAPA HiFi HotStart DNA polymerase, 1.5 μL barcode F (10 pM), 1.5 μL barcode R (10 pM), 5 μL purified product, and 10.75 μL retinoblastoma. The purified product was amplified by PCR using primers, where the barcode is an eight-base sequence unique to each sample. Denaturation, annealing, elongation, and cycling were the same as the first PCR amplification. The amplicons were subsequently purified by AMPure XP beads to clean up the final library before quantification. Finally, purified amplicons were pooled in equimolar and paired-end sequences (2 × 250) on an Illumina MiSeq platform according to the standard protocols.

2.4. Bioinformatics analysis of sequencing data

Fast length adjustment of short reads was used to merge paired-end reads from next-generation sequencing [15]. Low-quality reads were filtered by fastq_quality_filter (−p 90 −q 25 −Q 33) in FASTX Toolkit 0.0.14, and chimera reads were removed by USEARCH 64-bit version 8.0.1517. The number of reads for each sample was normalized based on the smallest size of samples by random subtraction. The final optimized sequence was obtained to ensure the reliability of the effective sequence used as operational taxonomic units (OTUs). OTUs were aligned by the Uclust algorithm with a 97% identity and taxonomically classified using the Silva16S rRNA database (https://www.arbsilva.de/documentation/release-128/). From the levels of phylum and genus, the dominant bacteria obtained by sequencing in each group were statistically analyzed. The α-diversity reflects a comprehensive indicator of microbial evenness and abundance in a single sample and mainly includes the abundance index Chao1, Shannon's index, and Simpson's index. In contrast, β-diversity is a comparative analysis of microbial community composition among different groups. Both α- and β-diversities were generated in the Quantitative Insights Into Microbial Ecology (QIIME) software and calculated based on weighted and unweighted Unifrac distance matrices [16]. Venn diagram selects OTUs with a similarity level of 97% and displays the number of OTUs shared by multiple groups, reflecting the similarity and overlap of environmental samples. The linear discriminant analysis (LDA) coupled with effect size measurement (LefSe) method was used to identify metagenomic biomarkers that exhibited statistically significant differential abundances among groups [17].

2.5. Statistical analysis

SPSS 22, GraphPad Prism7, and QIIME were used for statistical analysis. The Chi-square test was used for categorical data. Quantitative values are expressed as the mean ± standard deviation ($M \pm SD$). Two-sample independent t-test and the Wilcoxon rank-sum test were used between the two groups. For multi-group comparison, one-way analysis of variance and the Kruskal–Wallis rank-sum test were used. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Study population characteristics

This study investigated the relationship between gallstone formation and bacteria in the bile, gallbladder mucosa, and feces of 21 gallstone patients (eight males and 11 females; age range: 32 – 73 years old). From the gallstone group, we obtained 13 gallstone specimens (S1 – S13), nine bile specimens (Z1 – Z9), 13 gallbladder mucosa specimens (N1 – N13), and 17 feces specimens (F1 – F17). Meantime, we collected 20 feces (HF1 – HF17) samples from the control group. We rejected three samples due to amplification failure; one from the gallstone specimens, one from the gallstone patients' feces specimens, and one from the healthy subjects' feces specimens. The average age and BMI of the patients in the gallstone group were higher than that of the control group ($P = 0.004$). There were no statistically significant differences in gender and cholesterol levels between the gallstone and control groups (Table 1).

3.2. Bacterial diversity of sample species under different sequencing quantities and OTUs dilution curve

In this study, we identified a total of 23427 OTUs (340 ± 93) based on the conventional criterion of 97% sequence similarity, with 4095 OTUs in gallstones, 3065 OTUs in bile, 4687 in gallbladder mucosa, 5203 OTUs in patients' feces, and 6377 OTUs in normal feces. There was no significant difference in the intestinal microbiota diversity based on the feces of the gallstone and control groups. There was also no statistical difference in the bacterial diversity between gallstones, bile, and gallbladder mucosa in the gallstone group ($P > 0.05$). The gut microbiota was reportedly diverse in gallstones ($P = 0.004$), bile ($P = 0.045$), and gallbladder mucosa ($P = 0.008$). In addition, the gut microbiota was more diverse in the gallstone group than the control group (Table 2).

When the number of sequences increased, the diversity index did not increase significantly, indicating that the number of sequences was sufficient to reflect the overall community structure (Figure 1). In addition, the increase in the number of sequences did not generate new OTUs.

Table 1. Clinical data of the gallstone and control groups

Clinical parameter	Group		*P*-value
	Gallstone	Control	
Gender (males/females)	8/13	11/9	0.278
Age (years)	52.8±14.4	40.1±12.4	0.004
BMI (kg/cm^2)	24.4±2.4	22.8±2.1	0.032
Cholesterol (mmol/L)	1.6±0.7	1.3±0.5	0.126

Notes: Gender and cholesterol were analyzed with a Chi-square test; age and BMI were analyzed with a two-sample independent t-test.
Abbreviation: BMI: body mass index

DOI: https://doi.org/10.36922/jctr.23.00118

Table 2. Microflora sequencing results of each sample type

Statistical parameters	Sample type				
	Gallstone	Bile	Gallbladder mucosa	Patients' feces	Normal feces
Total number of sequences	1818953^{+}	1324611^{+}	2089573^{+}	2163367$^{\#}$	3033379$^{\#}$
Mean number of sequences	151579±80025	147179±62622	160736±58717	135210±56672	159651±40231
Total OTUs	4095^{+}	3065^{+}	4687^{+}	5203$^{\#}$	6377$^{\#}$
Mean OTUs	341±136	340±124	361±118	325±59	335±39
Chao1	434±131^{+}	416±119^{+}	445±120^{+}	418.15±51.50$^{\#}$	411.18±49.15$^{\#}$
Shannon's index	3.77±1.66^{+}	4.08±1.91^{+}	3.75±1.68^{+}	4.87±0.93$^{\#}$	5.17±0.64$^{\#}$
Simpson's index	0.71±0.19*$^{+}$	0.74±0.23*$^{+}$	0.69±0.18*$^{+}$	0.89±0.08$^{\#}$ *	0.92±0.06$^{\#}$

Notes: $^{\#}P > 0.05$ between gallstone, bile, and gallbladder mucosa; $^{+}P > 0.05$ between patients' feces and normal feces; $*P < 0.05$.
Abbreviation: OTU: Operational taxonomic unit

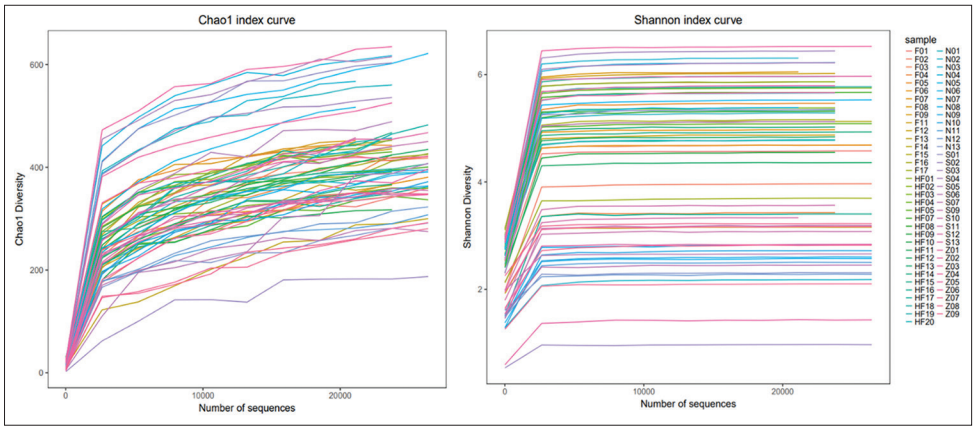

Figure 1. Operational taxonomic unit dilution curves.

3.3. Variable regions (V3-V4) of the bacterial 16S rRNA gene and bacterial community sequencing

Sequencing of V3-V4 fragments of the 16s rRNA gene yielded a total of 10,429,883 sequences with a mean ± standard deviation of 151 158 ± 57 813 from the gallstone, bile, gallbladder mucosa, and fecal samples (Table 2). The raw sequence reads were deposited in National Center for Biotechnology Information (NCBI) under Bioproject (accession no.: PRJNA 929661).

3.4. Comparative metagenomic analysis between gut and biliary tract at the phylum and genus level

The composition and diversity of bacteria at the genus level are often used to reflect changes in the microenvironment of specific human body parts [18]. Accordingly, we structurally analyzed each group of bacteria at the phylum and genus levels (Tables 3 and 4). At the phylum level (Table 3), Proteobacteria, Firmicutes, Bacteroidetes, Actinobacteria, Verrucomicrobia, Fusobacteria, Gemmatimonadetes, and Cyanobacteria reported no statistical difference in gut microbiota diversity between the gallstone and control groups (i.e., in the fecal samples) ($P > 0.05$). However, Proteobacteria, Firmicutes, and Bacteroidetes reported statistical differences among gallstone, bile, gallbladder mucosa, and fecal samples in the gallstone group.

At the genus level (Table 4), in comparison with the control group, the gallstone group displayed a decreased abundance of *Achromobacter*, *Faecalibacterium*, and *Lachnospira* ($P < 0.05$) and an increased abundance of *Enterococcus* ($P < 0.05$), while the other genera reported no statistical differences ($P > 0.05$). In the gallstone group, the abundance of *Achromobacter* was significantly higher in the biliary tract (including gallstones, bile, and gallbladder mucosa) than in the intestinal tract. In addition, the abundance of *Bacteroides*, *Faecalibacterium*, *Lachnoclostridium*, and *Subdoligranulum* in the gallstone group was significantly lower in the biliary tract (including gallstones, bile, and gallbladder mucosa) than in the intestinal tract. The abundance of *Enterococcus* was significantly higher and the abundance of *Parabacteroides* was significantly lower in the gallstone and bile specimens than in the fecal specimens of the gallstone group.

Pairwise comparison of the gallstone, bile, gallbladder mucosa, and fecal samples in the gallstone group revealed a significantly higher abundance of *Acinetobacter* in the biliary tract (including gallstones, bile, and gallbladder mucosa) and a significantly lower abundance of *Bacteroides*, *Faecalibacterium*, *Lachnoclostridium*, and *Subdoligranulum* in the intestinal tract (Figure 2).

In the gallstone group, the abundance of Proteobacteria was significantly higher and the abundance of *Firmicutes* and

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Table 3. Bacterial composition and diversity among sample types at the phylum level.

Phylum	Sample type (%)				
	Gallstone	Bile	Gallbladder mucosa	Patients' feces	Normal feces
Proteobacteria	83.68^{y}	89.53^{y}	90.78^{y}	6.51x^{y}	3.74^{x}
Firmicutes	16.75±21.55^{y}	13.33±15.56^{y}	9.85±13.37^{y}	36.20±16.52^{xy}	44.21±11.79^{x}
Bacteroidetes	6.11^{y}	3.76^{y}	2.04^{y}	44.13^{xy}	50.56^{x}
Actinobacteria	2.27	1.85	2.12	0.98^{x}	1.10^{x}
Verrucomicrobia	0.15	0.11	0.17	0.01^{x}	0.00^{x}
Fusobacteria	0.01	0.03	0.02	0.00^{x}	0.00^{x}
Gemmatimonadetes	0.05	0.00	0.12	0.00^{x}	0.00^{x}
Cyanobacteria	0.00	0.00	0.00	0.00^{x}	0.00^{x}

Notes: $^{x}P > 0.05$ between gallstone, bile, gallbladder mucosa, and patients' feces; $^{y}P < 0.05$ between patients' feces and normal feces

Table 4. Bacterial composition and diversity among sample types at the genus level.

Genus	Sample type (%)				
	Gallstone	Bile	Gallbladder mucosa	Patients' feces	Normal feces
Achromobacter	34.97^{m}	0.00^{m}	78.77^{m}	0.00z^{m}	0.29^{z}
Bacteroides	4.84^{n}	2.58^{n}	1.43^{n}	32.40^{n}	32.86
Escherichia/Shigella	0.76	0.28	0.24	0.86	0.18
Faecalibacterium	1.26^{n}	0.72^{n}	0.46^{n}	6.52z^{n}	10.15^{z}
Prevotella	0.11	0.21	0.08	0.84	0.45
Acinetobacter	0.12	0.12	0.09	0.00	0.01
Lachnospira	0.08	0.17	0.15	0.43^{z}	3.24^{z}
Lachnoclostridium	0.20^{n}	0.16^{n}	0.08^{n}	1.10^{n}	1.80
Blautia	0.16	0.27	0.14	1.62	1.03
Megamonas	0.14	0.07	0.09	0.05	0.02
Subdoligranulum	0.13^{n}	0.08^{n}	0.11^{n}	0.74^{n}	1.61
Enterococcus	0.19^{M}	0.04^{M}	0.14	0.02z^{M}	0.00^{z}
Bifidobacterium	0.16	0.28	0.13	0.30	0.57
Parabacteroides	0.40^{N}	0.10^{N}	0.10	0.82^{N}	0.76
Eubacterium	0.25	0.48	0.24	0.80	0.40

Notes: $^{z}P < 0.05$ between patients' feces and normal feces; $^{m}P < 0.05$ denote significantly higher % than in the intestinal tract; $^{n}P < 0.05$ denote significantly lower % than in the intestinal tract; $^{M}P < 0.05$ denote significantly higher % than in patients' feces; NP < 0.05 denote significantly lower % than in patients' feces

Bacteroidetes was significantly lower in the biliary tract than in the intestinal tract (Figure 3A). Likewise, the abundance of *Enterococcus* was significantly higher and the abundance of *Parabacteroides* was significantly lower in the gallstone and gallbladder mucosa samples than in the intestinal tract (Figure 3B). There was no statistical difference between bile and gut microbiota, and the abundance of *Prevotella* was significantly lower in the gallbladder mucosa of patients with gallstones than in the gut microbiota. We found no statistical difference in the other microflora structures between the biliary and intestinal tracts in patients with gallstones.

3.5. Beta diversity analysis and Venn plots

Principal coordinate analysis (PcoA analysis) was conducted based on the Bray-Curtis algorithm to validate the above findings (Figure 4). Between principal coordinate (PC) 1 and PC2, the diversity of the gut and biliary microbiota of patients in the gallstone and control groups was relatively similar. However, the diversity between PC2 and PC3 was relatively similar for some gut and biliary microbiota. The bile and gallbladder mucosa shared 757 OTUs, accounting for more than 90% (i.e., 90.1%, 93.9%, and 91.6%) of each group (i.e., between PC1 and PC2; PC1 and PC3; and PC2 and PC3). The gut microbiota of patients in the gallstone and control groups shared 607 OTUs, accounting for more than 85% of each group (87.8% and 95.0%, respectively). The five sample types shared 541 OTUs, accounting for more than 60% in each group (i.e., 65.6% in gallstone, 64.4% in bile, 67.1% in gallbladder mucosa, 78.3% in feces of the gallstone group, and 84.7% in feces of the control group) (Figure 5). The Venn plot selected OTUs with a similarity of 97% (or higher) and displayed the mutually shared number of OTUs between multiple groups, reflecting the similarity and overlap of the environmental samples.

3.6. Characteristic bacteria of the biliary and intestinal tract in gallstone patients and healthy subjects

LDA was used to reduce data dimensionality and evaluate the different abundance of each bacteria species [17]. Species with LDA values greater than the set threshold were regarded as biomarkers with statistical differences. It is generally believed

DOI: https://doi.org/10.36922/jctr.23.00118

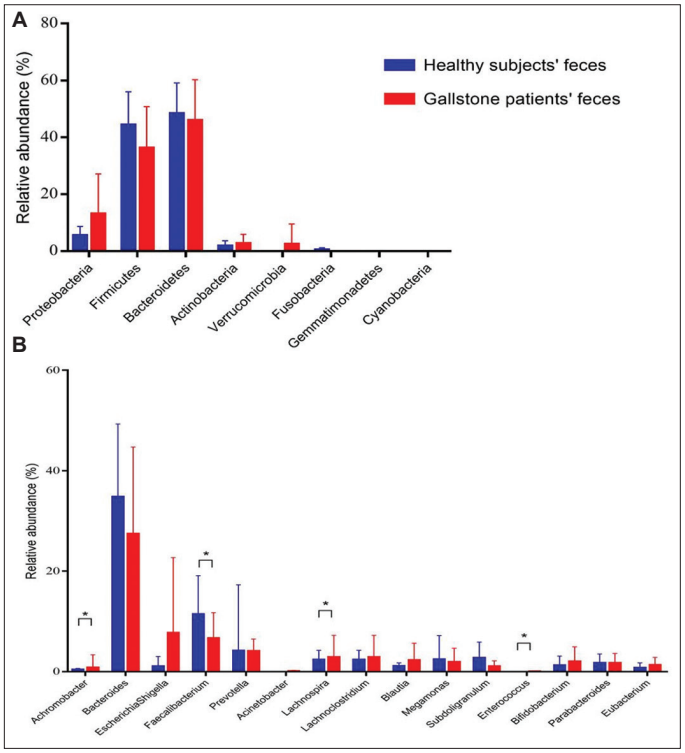

Figure 2. Relative abundance of bacterial flora at the (A) phylum and (B) genus levels between gallstone patients' feces and healthy subjects' feces specimens. Blue: healthy subjects' feces; red: gallstone patients' feces; *$P < 0.05$.

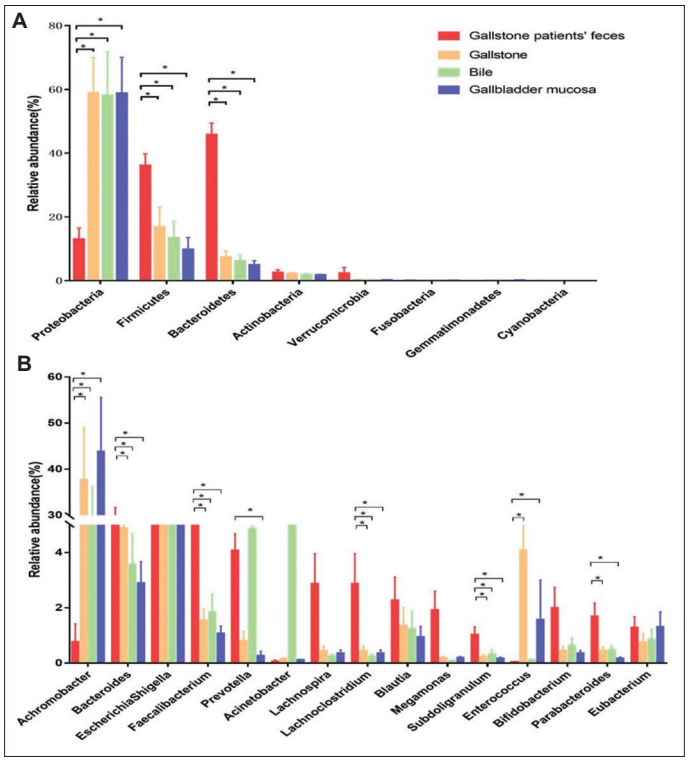

Figure 3. Relative abundance of bacterial flora at the (A) phylum and (B) genus levels between the biliary and intestinal tracts of gallstone patients. Blue: gallbladder mucosa; green: bile; orange: gallstone; red: gallstone patients' feces; *$P < 0.05$.

that LDA >3 indicates a significant difference. However, given the large variety of bacteria in the bile, gallbladder mucosa, and fecal samples from the gallstone and control groups, LDA >4 was adopted as the threshold value for screening characteristic bacteria (Figure 6). We found that in the gallstone group, *Prevotella* had LDA >4 in the fecal samples; Gamma proteobacteria, Pseudomonadales, *Moraxellaceae*, and *Acinetobacter* had LDA >4 in the bile sample; Proteobacteria, *Betaproteobacteria*, and *Burkholderiales* had LDA value >4 in the gallbladder mucosa sample. None of the bacteria had LDA >4 in gallstone samples, while the representative *Bacilli*, *Lactobacillales*, *Enterococcus*, and *Enterococcaceae* had LDA >3. Bacteria species with LDA >4 in the feces of the control group included *Bacteroidia*, *Bacteroidales*, *Bacteroidaceae*, *Bacteroides*, *Clostridia*, *Clostridiales*, *Firmicutes*, and *Ruminococcaceae* (Figure 6).

4. Discussion

Gut microbiota studies have gained momentum in recent years. The human intestine is colonized by over 100 trillion bacteria, involved in many body activities. Intestinal dysbiosis has been associated with various human diseases, such as kidney stones, obesity, diabetes, osteoporosis, and polycystic ovary syndrome [19]. The relationship between gallstones and gut microbiota has gradually become a research hotspot. Given that the gut microbiota is subject to many potential influencing factors, strict inclusion and exclusion criteria were established for this study. For instance, we excluded patients with severe bacteremia, sepsis, and a history of antibiotic or probiotic use in the past 3 months [20]. Moreover, we excluded patients with serious comorbidities (e.g., metabolic diseases) [21], prior use of somatostatin and other drugs affecting gallstone formation [22], history of intestinal surgery [23], and pregnant women or long-term contraceptive users. Due to the strict exclusion criteria, the number of samples enrolled in this study was limited. In this study, we studied the composition of bacterial communities in gallstones, bile, gallbladder mucosa, and intestinal samples from 21 gallstone patients, as well as the gut of 20 normal individuals. High-throughput sequencing was used to sequence V3-V4 fragments of the bacterial 16S rRNA gene. The total number of sequences obtained was 10 429 883 from 72 samples for subsequent statistical analysis.

This study found no significant difference in the abundance and diversity of gut microbiota between patients in the gallstone and control groups, consistent with previous literature [18]. However, studies have reported a decrease in intestinal microbial diversity and the abundance of the genus *Roseburia* [24]. This discrepancy in findings warrants further multicenter studies with larger samples to validate and evaluate the robustness of our findings. In comparison to the control group, the gallstone group reported a decreased abundance of *Achromobacter*, *Faecalibacterium,* and *Lachnospira* and an increased abundance of *Enterococcus*. Research has found that *Enterococcus* can shorten the nucleation time of cholesterol crystals in simulated bile and promote nuclear activity [25],

DOI: https://doi.org/10.36922/jctr.23.00118

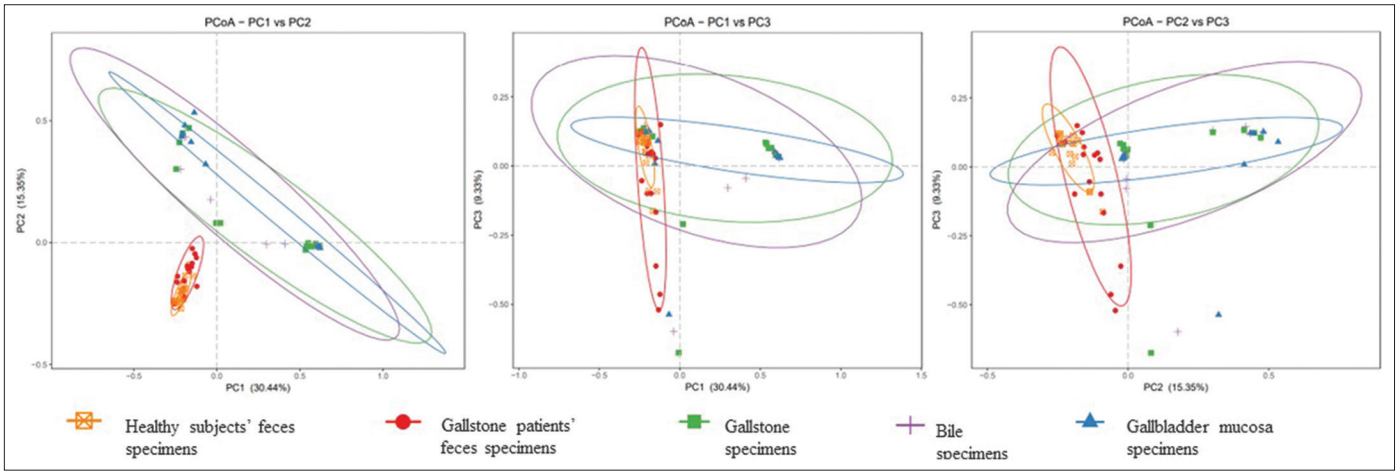

Figure 4. Principal coordinate analysis (PcoA) based on the Bray-Curtis algorithm between principal coordinate (PC) 1 and PC2, PC1 and PC3, and PC2 and PC3.

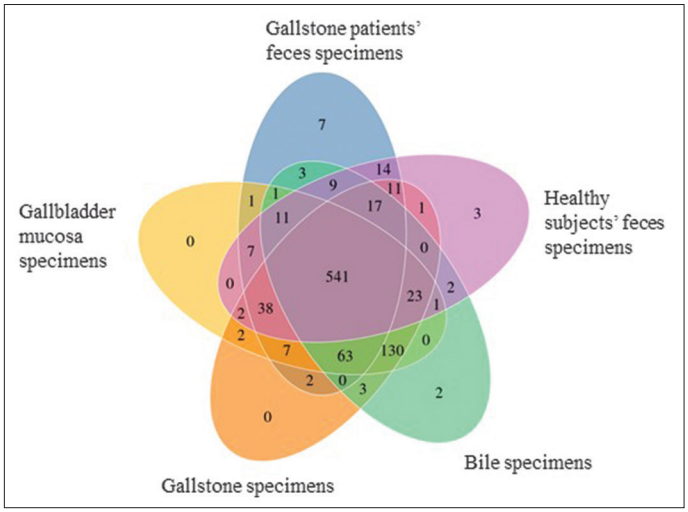

Figure 5. Venn plot of operational taxonomic units between the sample types.

representing a potential mechanism for the increased abundance of *Enterococcus* in patients with gallstones. Studies have also demonstrated that Proteobacteria in the intestine of patients with gallstones were prone to bacterial overgrowth, which was also observed in a wide range of pathogenic microorganisms, such as *Escherichia coli*, *Salmonella*, *Vibrio,* and *Helicobacter* [18]. Animal experiments found that cholesterol stones were formed in mice fed with a lithogenic diet, and the gut microbiota of *Firmicutes* and *Bacteroidetes* was significantly reduced [26]. Taken together, the above findings suggested that gut microbiota disorders are common in patients with gallstones.

We found that the bacterial diversities of gallstones, bile, and gallbladder mucosa were significantly different from the gut microbiota (based on Simpson's index), indicating greater diversity in the gut microbiota than in the biliary microbiota of patients with gallstones. Several studies have concluded that the average biodiversity of bile microorganisms decreases in patients with recurrent choledocholithiasis [27], suggesting that the decreased biodiversity may weaken the elasticity of natural ecosystems and increase the possibility of serious ecosystem degradation [28]. Nonetheless, it has been observed that the bacterial diversity of the biliary tract was significantly higher than that of the intestinal tract [18]. This discrepancy may be caused by the significant differences in biliary microbiota between individuals [29].

At the phylum level, the abundance of Proteobacteria was significantly higher and the abundance of *Firmicutes* and *Bacteroidetes* was significantly lower in the biliary tract than in the intestinal tract, similar to existing research. Proteobacteria can participate in oxidative stress and are a potential microbial diagnostic marker of epithelial dysfunction [30]. The formation of gallstones is also related to epithelial dysfunction [31], which can explain the high abundance of Proteobacteria in patients with gallstones to a certain extent. Increasing evidence corroborates that the number of Proteobacteria in bile from patients with recurrent choledocholithiasis is significantly higher than in patients without cholelithiasis [32].

In this study, LefSe analysis identified many types of Proteobacteria with LDA >4 in the biliary tract and *Firmicutes* and *Bacteroidetes* with LDA value >4 in the intestine of the control group. This finding further validated the differences in bacterial community structure at the phylum level. At the genus level, the abundance of *Acinetobacter* (belonging to Proteobacteria), *Bacteroides*, *Faecalibacterium*, and *Lachnoclostridium* was significantly higher in the biliary tract than in the intestinal tract. The abundance of *Subdoligranulum* was significantly lower than in the gut. Several studies have revealed that *Acinetobacter* can produce *β*-glucuronidase, which hydrolyzes bilirubin glucuronic acid to produce free bilirubin that combines with free calcium ions to form gallstones [25,33].

This study found a significant increase in *Prevotella* in the intestines of patients with gallstones compared to the biliary microbiota. In LefSe analysis, *Prevotella* had LDA >4 in the feces of patients with gallstones, suggesting that *Prevotella* can be used as a biomarker for bacterial dysregulation in patients with gallstones. In this regard, a meta-analysis of 1791 patients demonstrated that *Prevotella* is involved in atherosclerosis [34],

DOI: https://doi.org/10.36922/jctr.23.00118

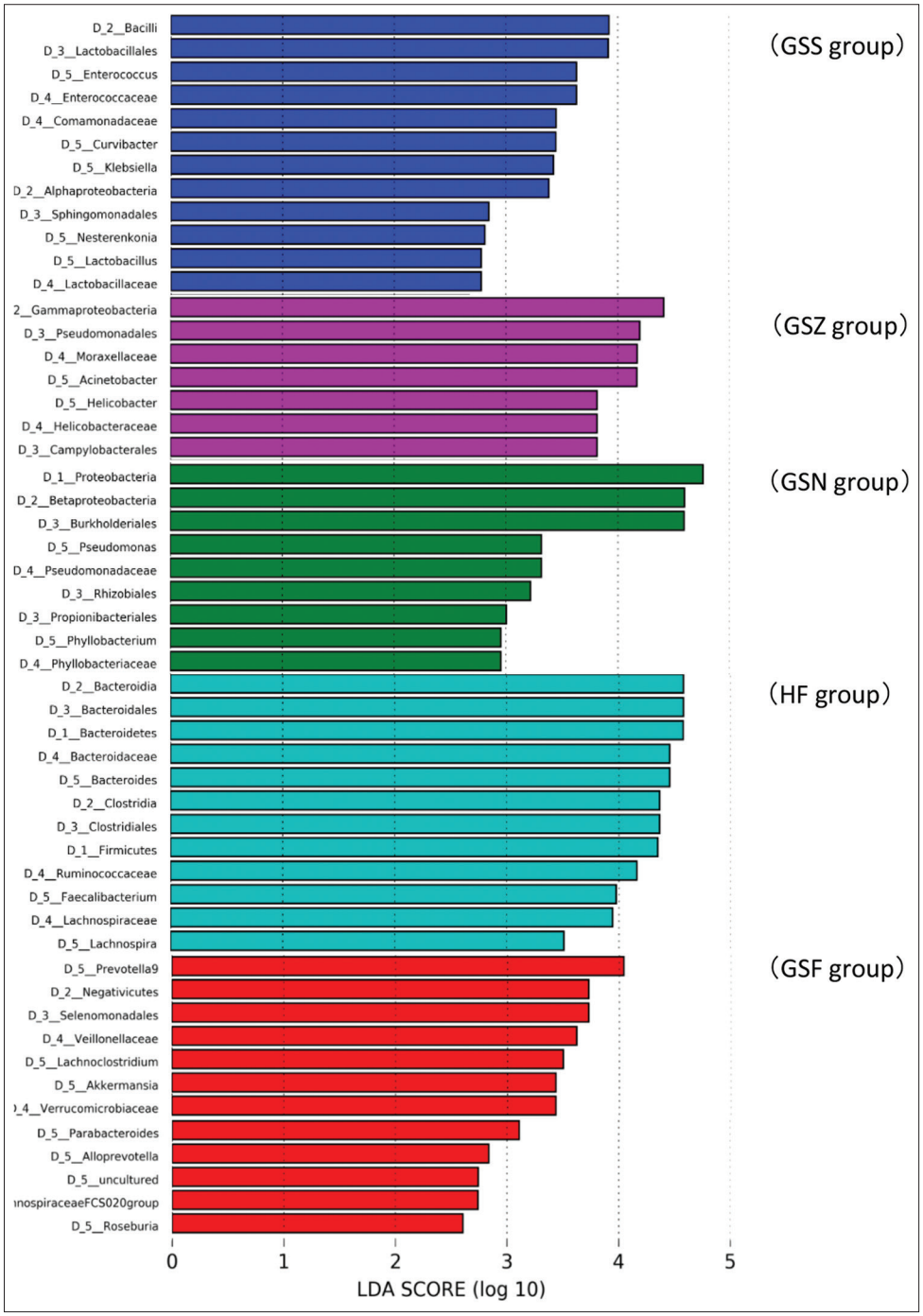

Figure 6. Histogram of LDA scores for differentially abundant genera in the gallstone, bile, gallbladder mucosa, gallstone patients' feces specimens, and healthy subjects' feces specimens. The cladogram was calculated by LDA and displayed according to effect size.
Abbreviations: GSF: Feces from the gallstone group; GSN: Gallbladder mucosa; GSS: Gallstone; GSZ: Bile; HF: Feces from the control group; LDA: Linear discriminant analysis.

and it is known that metabolic factors related to atherosclerosis are closely associated with gallstone formation [12].

In this study, the PC1 versus PC2 plots displayed relatively similar gut and biliary microbiota between the gallstone and control groups, suggesting the specificity of the biliary tract and gut microbiota. However, the PC2 versus PC3 plot revealed some diversity between the gut and biliary microbiota, indicating a certain degree of overlap between the intestinal and biliary microbiota. The OTUs of the biliary and intestinal tract were visualized with a Venn plot, and the results reflected the overlapping OTUs among the samples, indicating that the biliary microbiota may be partly derived from a retrograde intestinal infection and providing novel insights into the source of biliary microbiota. Subsequent PCoA analysis validated this finding,

DOI: https://doi.org/10.36922/jctr.23.00118

and elucidating the diversity of biliary and gut microbiota in gallstone diseases may facilitate the prognosis and management of gallstones.

Nonetheless, there were several limitations in this study, such as the small sample size. Cholecystectomy and endoscopic operations are invasive procedures, and this study did not include bile samples from the control group for comparisons. This study selectively excluded the research subjects, resulting in self-selection and sample-selection biases, thereby limiting an accurate representation of the population.

5. Conclusion

The biliary tract (e.g., gallstones, bile, and gallbladder mucosa) and gut microbiota of patients with gallstones exhibited specific changes compared to healthy individuals, providing novel insights into the source of the biliary tract flora and the formation of gallstones. Patients with gallstones had an obvious microbiota imbalance at the genus level. The diversity of gut microbiota was higher than that of biliary microbiota among gallstone patients. *Enterococcus* abundance was higher in the intestines of patients with gallstones than in healthy individuals. *Prevotella* and Proteobacteria may serve as biomarkers for dysbacteriosis in patients with gallstones as alterations of the biliary and gut microbiota may be related to the formation of gallstones. The identification of characteristic microbiota warrants further studies on gene function annotation and metabolic pathways to explore the pathogenesis of gallstones and its significance in gallstone prevention and treatment. In addition, our research provided new ideas for exploring the feasibility of fecal microbiota transplantation for the treatment of gallstone disease. Based on the specific microbiota of gallstone patients identified in this study, we will conduct further studies on bile acid metabolomics to (i) explore the correlation between gut microbiota imbalance and bile acid metabolism, (ii) identify potential metabolic pathways and targets, and (iii) establish a network relationship between gut microbiota imbalance and gallstones formation. In summation, fecal microbiota transplantation could reconstruct new gut microbiota and evaluate the treatment of extraintestinal diseases.

Acknowledgments

We thank the study team, including study coordinators, operating nurses, physicians, data management personnel, and other support staff, for the practical arrangements of the study, as well as colleagues for their assistance with transporting the study materials.

Funding

This work was supported by the Digestive Medical Coordinated Development Center of the Beijing Hospitals Authority (Grant number: XXT14).

Conflict of Interest

The authors have no relevant financial or non-financial interests to disclose.

Ethical Approval and Consent to Participate

Ethical approval was obtained from the Institutional Research Ethics Committee of Beijing Tiantan Hospital, Capital Medical University (KY2020-032-02). We obtained informed consent from human patients before their participation by signing an informed consent form.

Consent for Publication

We obtained informed consent from human patients before their participation by signing an informed consent form.

Availability of Data

The datasets generated during the present study are available in the NCBI database. The raw sequence reads of this study were deposited at NCBI under Bioproject with the accession number PRJNA 929661.

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DOI: https://doi.org/10.36922/jctr.23.00118

Journal of Clinical and Translational Research 2024; 10(3): 191-200

Journal of Clinical and Translational Research

Journal homepage: http://www.jctres.com/en/home

ORIGINAL ARTICLE

Drug therapy problems related to cardiovascular agents and associated factors among heart failure patients: a prospective observational study of a tertiary inpatient setting in Uganda

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ARTICLE INFO

Article history:
Received: December 13, 2023
Accepted: February 29, 2024
Published Online: May 28, 2024

Keywords:
Heart failure
Drug therapy problem
Cardiovascular agents

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ABSTRACT

Background: Heart failure (HF) is a clinical syndrome that is treated with multiple medications, putting patients at risk of drug therapy problems (DTPs). DTPs are a great concern in health care due to their significant impact on morbidity, mortality, and higher costs associated with health care.
Aims: This study aimed to assess the prevalence and factors associated with DTPs related to cardiovascular agents among HF patients hospitalized at the Mbarara Regional Referral Hospital.
Methods: A prospective observational study was conducted among hospitalized HF patients from November 2021 to January 2022. A total of 118 patients diagnosed with HF were recruited. Patient file reviews and interviewer-administered questionnaires were used for data collection. Univariate and multivariate logistic regressions were employed to determine factors associated with the DTPs.
Result: A total of 118 HF patients with a median age of 43 years were included in this study. Among them, 57 participants experienced a total of 81 DTPs with a prevalence of 48.3%. "Dosage too high" was the most common DTP (23, 28.3%) followed by "needs additional drug therapy" (22, 27.2%). Hospitalized HF patients aged 60 years and above (adjusted odds ratio [AOR]: 4.1; 95% confidence interval [CI]: [1.5 – 10.3]; $P = 0.012$) and taking more than five medications during their hospital stay (AOR: 2.92; 95% CI: [1.5 – 7.6]; $P = 0.029$) were significantly associated with experiencing at least one DTP.
Conclusion: Almost half of the hospitalized HF patients experienced at least one DTP during their hospital stay. "Dosage too high" and "needs additional drug therapy" were the most common DTPs. Hospitalized HF patients with poly-pharmacy (taking more than five cardiovascular agents) and aged above 60 were more likely to have DTPs. It is noted that patient groups who are at risk require follow-ups to improve the treatment outcome, and incorporation of clinical pharmacy service could be part of the solution.
Relevance for Patients: This study identifies the factors associated with the development of DTP to facilitate the development and implementation of prevention strategies for the commonly identified DTPs.

1. Introduction

Heart failure (HF) is a clinical syndrome characterized by functional or structural impairments of the heart leading to the inability of the organ to fill with or pump a sufficient amount of blood to meet the metabolic needs of the body [1,2]. HF can be managed by lifestyle modification and lifelong therapy with multiple medications [3]. There has been a consistent increase in the number of medications used in HF patients, but this has been

DOI: https://doi.org/10.36922/jctr.23.00137

associated with detrimental health outcomes, such as frequent hospitalization and drug-related problems [4,5].

Over 40% of newly diagnosed cardiovascular disease (CVD) patients are HF patients [6]. The incidence and prevalence of CVD in Africa are increasing, and CVD is predicted to overtake communicable disease as the most common cause of death in a few decades, especially in sub-Saharan Africa (SSA) [7]. The impact of CVDs, including HF, is more prominent in the working class from low- and middle-income countries, like Uganda [8,9]. However, there is a lack of population-based incidence and prevalence of HF studies in SSA, including Uganda. It was reported that HF is responsible for 9.4 – 42.5% of all medical admissions and 25.6 – 30.0% of admissions into the cardiac units [10].

A drug therapy problem (DTP) can be defined as any undesirable drug treatment-related event experienced by a patient, which potentially interferes with the desired goals of therapy and requires professional intervention to resolve [11]. DTPs can be classified using validated tools, like Cipolle's [11], Pharmaceutical Care Network Europe [12], and APS-Doc [13]. Common DTPs can be classified accordingly as: "dosage too low," "adverse drug reaction," "needs additional drug therapy," "ineffective drug," "unnecessary drug therapy," "dosage too high," and "non-adherence" [11]. DTPs are common in HF patients as HF treatment involves multiple medications (poly-pharmacy), coupled with its risk factors, i.e., comorbidities, advanced age, and poor continuity of care [3,14–19].

The prevalence of DTPs among HF patients is reportedly 29.8 – 88.66% [20,21]. HF patients are vulnerable to DTPs, which raises their risk of mortality and morbidity [22]. DTPs are related to clinical outcomes, health-care costs, and quality of life of CVD patients [23]. Reducing health-care costs, mortality, and hospitalization and improving quality of life can be achieved through optimized drug therapy to prevent DTPs [24].

The increase in HF prevalence in SSA [25] and cardiovascular drug-related DTPs warrant further investigation into the prevalence of DTPs and their associated factors in HF patients for a better treatment outcome. Given the lack of data regarding cardiovascular drug-related DTPs in HF patients and undefined factors associated with DTPs, the present study aimed to determine the prevalence and factors associated with cardiovascular drug-related DTPs among HF patients hospitalized at the Mbarara Regional Referral Hospital (MRRH), Mbarara, Uganda.

2. Methods

2.1. Study design

This study was a prospective observational study conducted among hospitalized HF patients at the MRRH medical and pediatric wards from November 2021 to January 2022. MRRH is a 600-bed tertiary hospital and is the largest referral center in southwestern Uganda, 280 km from the capital Kampala. The hospital serves a population of over four million people in its catchment area comprising 13 districts of southwestern Uganda. The medical inpatient ward comprises 50 beds with an estimated monthly admission of 300 patients.

The study population included all inpatient HF patients who were hospitalized at MRRH during the study period. The inclusion and exclusion criteria were:

(i) Inclusion criteria: All newly diagnosed or known HF patients who were hospitalized for any reason at MRRH (internal medicine for adult and general pediatric wards) during the study period and were willing to participate in the study.

(ii) Exclusion criteria: Patients who were critically ill and/or in intensive care units.

2.2. Sample size determination

The sample size (n) was calculated using a single proportion formula;

$$n = Z^2\, p\, (1–p)/w^2 \quad \text{(I)}$$

Where n is the required sample size, p is the prevalence of the DTP, w is the tolerated margin of error (5%), and Z is the level of confidence (i.e., 1.96 at 95% confidence interval [CI]). The prevalence of DTP among HF patients from a previous study in Ethiopia was 91.3% [26]. Since the study settings were similar, we used 91.3% as p of DTP among hospitalized HF patients, with a 0.05 significance level at a 95% CI (i.e., p: 91.3%; w: 0.05; Z: 1.96 [95% CI]).

Using the above formula, the number of patients to be included in the study was 122. The *post hoc* power was 69 – 96% for variables included in the logistic regression analysis, with a generally acceptable level of type II errors.

A consecutive sampling technique was used during the study period, and data collection was continued for 3 months (November 2021 – January 2022) until the required sample size was achieved.

2.3. Data quality control

Pre-testing of the data collection tool was conducted using 10 patients who met the inclusion criteria. This was performed to identify any challenges or issues with the tool, allowing for necessary modifications and refinements to be made thereafter. The principal investigator (E.A.S.) selected and recruited research assistants, and training on data collection protocols and ethical considerations was given before the study's commencement. The data collection tool was translated to Runyankole (the local language in western Uganda) by a professional and then the results were back-translated to English to check for consistency.

2.4. Data collection

All patients who presented to the medical and pediatric inpatient units with a diagnosis of HF were subjected to preliminary screening and assessed for eligibility as potential study participants. The research assistants and the principal investigator enrolled patients as study participants upon voluntarily consenting to participate by writing. Assent forms were prepared for the pediatric patients and their parents, and

DOI: https://doi.org/10.36922/jctr.23.00137

the data were collected after voluntary consent. The study aim was explained on enrollment.

Questionnaire-based interviews were conducted among eligible participants to obtain participants' baseline socio-demographics, past medical history, medication use, social drug use, and any known drug allergies. A data collection form was used to obtain data from patients' medical files. Patients' vital signs were taken daily and recorded. Laboratory and diagnostic investigations and current medication use were recorded daily.

Each drug therapy given during the hospital stay was evaluated for appropriateness, effectiveness, and safety based on Ugandan Clinical Guidelines (UCG, 2016), Up-To-Date (2019) version 3.12.0.44, and HF treatment guidelines. The main outcome measure refers to DTP, which was determined if any undesirable event was experienced by a patient that involves drug treatment, potentially interfering with achieving the desired goals of therapy [11]. In addition, poly-pharmacy was determined when a patient consumes five or more medications daily.

DTPs that were identified were categorized according to the Cipolle *et al.* classification system [11]. When a patient had more than one DTP during follow-up, each DTP was classified and counted separately**.** Lexicomp software was used to detect potential drug–drug interaction (DDI), and it was recorded as clinically significant when the interaction was rated as C, D, and X as per the Lexicomp drug interaction checker. The primary investigator (E.A.S.) classified the common drugs used according to the World Health Organization (WHO)-Anatomical Therapeutic Chemical (ATC) classification method [27]. The above-mentioned tools are standard to assess DTP and valid to use in our study based on previous studies in a similar setting. The Cipolle *et al.* DTP classification system [11] is summarized as follows:

(i) "Unnecessary drug therapy" defines a situation where drug therapy is unnecessary as the patient does not have a clinical indication for drug therapy; the use of multiple drug products for a condition that requires only a single drug; when nondrug therapy is more appropriate; addiction or recreational drug use; or treating avoidable adverse reactions [11].

(ii) "Needs additional drug therapy" defines a situation where a patient requires additional medication either for an untreated condition or to prevent a new medical condition [11].

(iii) "Ineffective drug" defines a situation where a different drug is needed after the patient is prescribed a drug that is not the most effective for the medical condition; the medical condition is refractory to the drug product; the dosage form of the drug product is inappropriate; the drug product is contraindicated in this patient; or the drug is not indicated for the condition [11].

(iv) "Dosage too low" defines a situation where the dosage is too low to produce the desired response in the patient; the dosage interval is too infrequent to produce the desired response; incorrect administration; a drug interaction that reduces the bioavailability of the active drug, resulting in lack of effectiveness in this patient; or the duration of the drug therapy is too short to produce the desired response [11].

(v) "Dosage too high" can be related to toxicity; the dosing frequency being too short for the patient; the duration of drug therapy being too long for a patient; or a drug interaction that increases the bioavailability of the active drug, resulting in toxicity in a patient [11].

2.5. Data analysis

All the statistical data analysis was performed using the Statistical Package for the Social Sciences (SPSS), version 21 (SPSS Inc., United States of America [USA]). The descriptive analysis of sociodemographic, clinical, and drug-related variables was presented as the median with the interquartile range (IQR) or percentage (%).

The prevalence of cardiovascular agent-related DTPs among hospitalized HF patients was calculated as follows:

$$\text{Prevalence}\ (\%) = \frac{\text{Number of cardiovascular agent} - \text{Related DTP patients}}{\text{Total number of patients}} \times 100\% \quad \text{(II)}$$

Univariate and multivariate logistic regressions were employed to determine the independent factors associated with DTPs. Variables were included in the model based on their significant association with DTP in previous studies. Multicollinearity between the independent variables was checked, indicating that there was no significant correlation between two the variables. Variables with $P < 0.25$ from the univariate analysis were included in the multivariate logistic regression. In the multivariate model, $p < 0.05$ was considered statistically significant.

3. Results

3.1. Recruitment and sociodemographic characteristics of participants

During the study period, 123 patients were screened for eligibility. Among these, five patients were excluded: three were unwilling to consent and two were discharged before an interview could be conducted; 118 HF patients were included in the final analysis. The median (IQR) age of the patients was 43 (20.75 – 69.25); 44 (37.3%) patients were elderly patients; 72 (61%) patients were females (Table 1).

3.2. Clinical characteristics and medication use of patients

A total of 56 (47.5%) patients were newly diagnosed with HF; 32 (27.1%) patients stayed in the hospital for more than 11 days; 93 (78.8%) of the patients had at least one comorbid condition; 75 (63.5%) of the patients were on poly-pharmacy; and 79 (66.9%) patients incurred a significant DDI among their medications (Table 2).

According to the ATC classification, anti-infective agents were used by 53 (44.9%) of the patients, followed by the use of alimentary tract and metabolism agents by 48 (40.6%) patients (Figure 1). Hypertension was the most common comorbid condition (37 [31.4%]), followed by kidney disease (23 [19.5%]) (Figure 2). All patients were using at least one cardiovascular agent.

DOI: https://doi.org/10.36922/jctr.23.00137

Table 1. Socio-demographic characteristics of hospitalized heart failure patients at the MRRH

Variable	*n* (%)
Age (years)	
≤18	26 (22)
19 – 59	48 (40.7)
≥60	44 (37.3)
Sex	
Male	46 (39)
Female	72 (61)
Educational status	
No formal education	53 (44.9)
Primary	53 (44.9)
Secondary and above	12 (10.2)
Occupation	
Unemployed	42 (35.6)
Self-employed/private business	71 (60.2)
Formally employed	5 (4.2)
History of alcohol use	33 (28.0)

Abbreviation: OTC: Over-the-counter.

Table 2. Clinical characteristics and medication use of hospitalized HF patients at the MRRH

Variable	*n* (%)
Status of HF	
New	56 (47.5)
Known	59 (52.5)
Previous hospital admission	78 (66.1)
Length of hospital stay (days)	
≤5	27 (22.9)
6 – 10	59 (50)
≥11	32 (27.1)
Total comorbidities	93 (78.8)
Number of comorbidities	
1	56 (60.2)
≥2	37 (39.8)
Major comorbid conditions	
Hypertension	37 (31.4)
Kidney disease	23 (19.5)
Anemia	18 (15.3)
Counseling on medication use	107 (90.7)
OTC medication use within the past 4 weeks	42 (35.6)
Herbal use within the past 4 weeks	52 (44.1)
Poly-pharmacy	75 (63.5)
Significant drug-drug interaction	79 (66.9)
Treatment affordability	18 (15.3)

Abbreviations: HF: Heart failure; MRRH: Mbarara Regional Referral Hospital; OTC: Over-the-counter.

3.3. Prevalence of DTPs related to cardiovascular drugs

Out of the 118 patients, 57 had at least one DTP during their hospital stay, corresponding to a prevalence of 48.3% (95% CI: 39 – 56.8%) (Figure 3). A total of 81 DTPs related to cardiovascular agents were identified among the 57 patients:

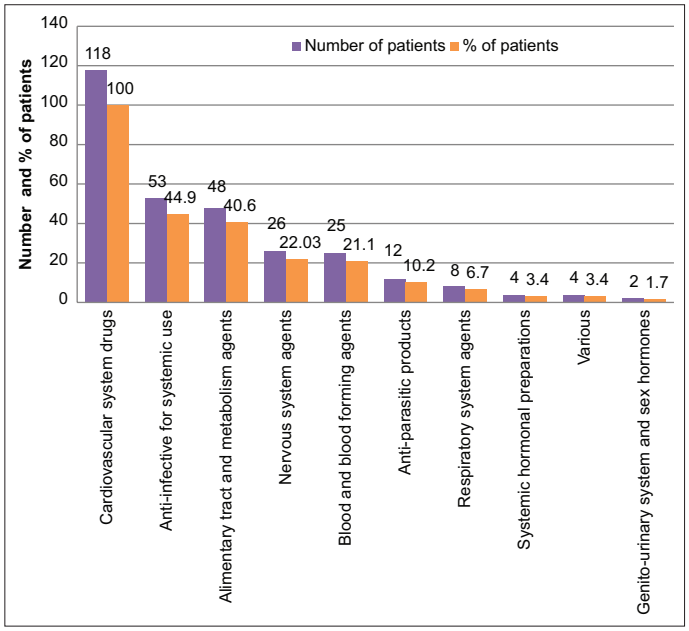

Figure 1. Common medications used among hospitalized heart failure patients at the Mbarara Regional Referral Hospital.

38 (66.7%) had one, 15 (26.3%) had two, 3 (5.2%) had three, and 1 (1.8%) had four DTPs.

3.4. Types and causes of DTPs related to cardiovascular agents

"Dosage too high" (23 [28.3%]) was the most common type of DTP related to cardiovascular agents, followed by "needs additional drug therapy" (22 [27.2%]) (Figure 4). For the "dosage too high" classification, the medication dose being too high was the most common cause, identified for 19 (23.5%) patients. For the "needs additional drug therapy" classification, an untreated condition was the most common, identified for 15 (18.5%) patients (Table 3).

3.5. Factors associated with the occurrence of DTPs

A total of 14 independent factors were analyzed using univariate logistic regression. Among these variables, age ≥60 years (crude odds ratio [COR]: 4.8; 95% CI: [2.1 – 10.6]; P = 0.003), gender, occupation, previous hospital admission, length of hospital stay >11 days (COR: 4.1; 95% CI: [1.4 – 10.7]; P = 0.012), comorbidity, herbal use within the previous 4 weeks, and poly-pharmacy were qualified for multivariate logistic regression analysis at P = 0.25 (Table 4).

According to the multivariate logistic regression, elderly patients >60 years old were four times more likely to experience DTPs related to cardiovascular agents compared to the pediatrics group (adjusted odds ratio [AOR]: 4.1; 95% CI: [1.5 – 10.3]; P = 0.012). Patients who were taking more than five medications during their hospital stay were three times more likely to experience DTPs related to cardiovascular agents than those who were taking less than five medications (AOR: 2.92; 95% CI: [1.5 – 7.6]; P = 0.029) (Table 4).

DOI: https://doi.org/10.36922/jctr.23.00137

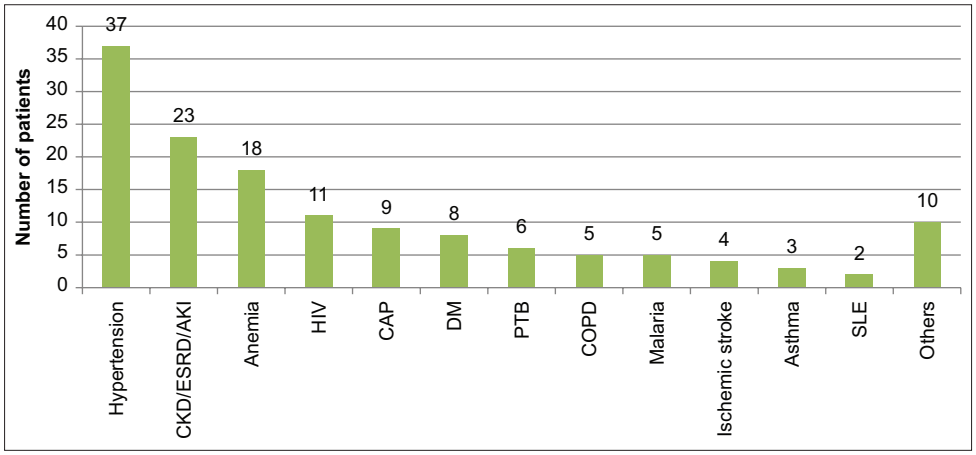

Figure 2. Common comorbid conditions among hospitalized heart failure patients at the Mbarara Regional Referral Hospital. The "others" category includes alcoholic liver disease, hypothyroidism, thyrotoxicosis, deep vein thrombosis, pharyngitis, interstitial lung disease, cholecystitis, peptic ulcer disease, cellulitis, and vitamin D deficiency.
Abbreviations: AKI: Acute kidney injury; CAP: Community-acquired pneumonia; CKD: Chronic kidney disease; COPD: Chronic obstructive pulmonary disease; DM: Diabetes mellitus; ESRD: End-stage renal disease; HIV: Human immunodeficiency virus; PTB: Pulmonary tuberculosis; SLE: Systemic lupus erythematosus.

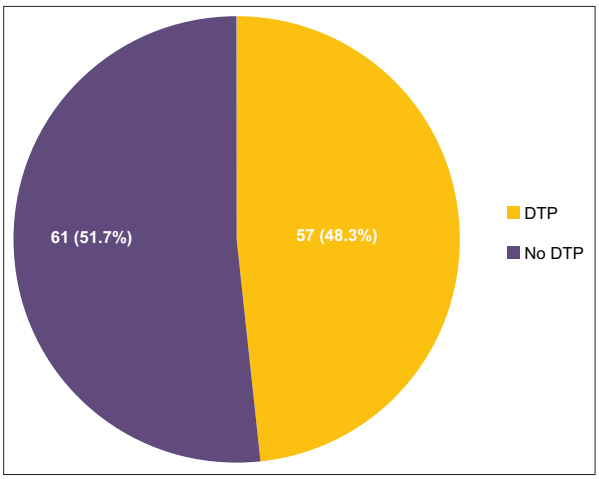

Figure 3. Prevalence of drug therapy problems related to cardiovascular agents among hospitalized heart failure patients at the Mbarara Regional Referral Hospital from November 2021 to January 2022.

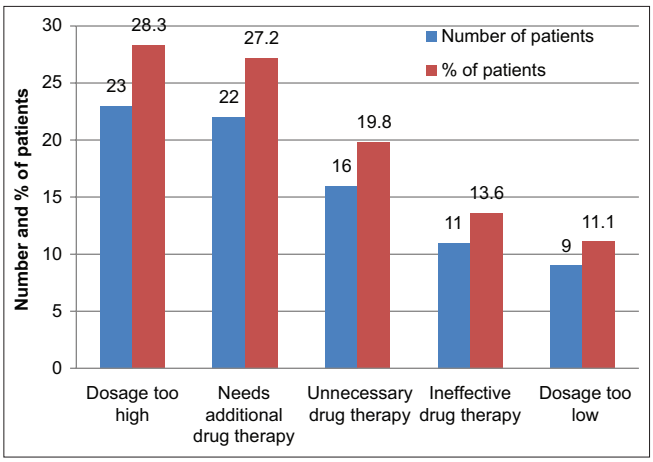

Figure 4. Types of drug therapy problems related to cardiovascular agents among heart failure patients hospitalized at the Mbarara Regional Referral Hospital from November 2021 to January 2022.

4. Discussion

The primary aim of our study was to assess the prevalence of DTPs related to cardiovascular agents among HF patients. Our findings revealed that 48.3% of the HF patients had at least one DTP related to cardiovascular agents during their hospital stay, indicating a significant prevalence of DTP among this patient population, which is comparable to the reported DTP prevalence of 39.3% in a previous study in India [22]. However, the prevalence of DTPs related to cardiovascular agents is lower than the reported prevalence in previous prospective observational studies conducted among hospitalized HF patients in Ethiopia, i.e., 91.3% [26] and 83.5% [28] in Jimma, 90.6% [29] in Bahir Dar, and 68.8% [30] at the Tikur Anbessa Specialized Hospital (TASH). The low prevalence of DTP in our study may be attributed to our DTP assessment method which only assessed the DTP related to cardiovascular agents used by the hospitalized HF patients, while the other studies [26,28-30] assessed the DTP related to all drugs used by the HF patients. A study conducted in Spain reported a high prevalence of DTP, but the prevalence of DTPs related specifically to HF medications was only 22% [31]. The discrepancy in prevalence with our findings could also be due to the different study populations, sample sizes, inclusion criteria, and study designs; the studies conducted in Ethiopia had a larger sample size and used a cross-sectional study design; the study at TASH [30] was conducted among HF patients who were in ambulatory care. Moreover, during this study, data collection period and clinical pharmacy residents were actively assessing and preventing DTPs among noncommunicable diseased patients in the medical ward of our study site. Our study highlights the need to prioritize the burden of DTPs and design an effective intervention to mitigate

DOI: https://doi.org/10.36922/jctr.23.00137

Table 3. Types of DTPs and their causes among hospitalized HF patients at the MRRH from November 2021 to January 2022

Type of DTP	Cause of DTP	*n* (%)
Needs additional drug therapy	Untreated condition	15 (18.5)
	Preventive drug needed	2 (2.5)
	Synergistic therapy	5 (6.2)
Unnecessary drug therapy	No valid medical indication	5 (6.2)
	Duplicate therapy	9 (11.1)
	To treat avoidable ADR	2 (2.5)
Dosage too low	Ineffective dose	3 (3.7)
	Dosing too infrequent	5 (6.2)
	Short duration of therapy	1 (1.2)
Ineffective drug therapy	More effective drug available	11 (13.6)
Dosage too high	Dose too high	19 (23.5)
	Drug interaction	3 (3.7)
	No dose adjustment for renal impairment	1 (1.2)

Abbreviations: HF: Heart failure; MRRH: Mbarara Regional Referral Hospital; DTPs: Drug therapy problems; ADR: Adverse drug reaction.

the associated problems. In addition, this study also warrants further research among CVD patients on the study site.

Multiple studies conducted among patients on cardiovascular agents revealed DTPs related to cardiovascular agents. A study conducted among Taiwanese HF patients [32] and in Barcelona [31] identified that the principal drugs associated with DTPs were cardiovascular agents, such as angiotensin-converting enzyme/angiotensin II receptor blockers (ACEI/ARBs), diuretics, spironolactone, and β-blockers.

The most common type of DTP related to cardiovascular agents identified in this study was "dosage too high" (28.3%). The common cause for "dosage too high" was the prescribed medication dose being too high for the hospitalized patients. This might be attributed to a lack of compliance with the standard and updated guidelines while selecting and dosing medications for HF patients. Moreover, it could be due to failure to assess the patient's organ functions (e.g., renal and hepatic functions), the patient's age and weight, and possible DDIs. On the contrary, studies [15,28,33] had reported that "dosage too low" (22 – 27.8%) was one of the common DTPs observed among patients who were using cardiovascular agents, whereas our finding reported that only 11.1% of DTPs related to cardiovascular agents were "dosage too low." Therefore, efforts should focus on minimizing drug dosing problems in HF patients by adhering to standard treatment guidelines and increasing the involvement of clinical pharmacists in deciding the dosing of drugs, i.e., considering the age and clinical condition of the patient.

The second most common DTP related to cardiovascular agents in this study was "needs additional drug therapy" (27.2%). This proportion is consistent with many studies involving CVD patients, i.e., 27.4 – 45.5% [15,26,28,33–36]. In the current study, the common cause for "needs additional drug therapy" was the presence of an untreated condition, leading patients to low quality of life, increased hospitalization, increased overall health-care cost, and death if undetected and resolved early [28]. This emphasizes the importance of ensuring the appropriate use of medication by adhering to national and evidence-based guidelines to optimize patient outcomes. Adverse drug reactions, another DTP, were reported in 59.3% of the same population [37].

The identification of associated factors for DTPs related to cardiovascular agents helps to identify the most susceptible patients who require close monitoring of drug therapy [15]. Multivariate logistic regression revealed that elderly patients >60 years old were four times more likely to be associated with DTPs related to cardiovascular agents compared to the pediatrics group (AOR: 4.1; 95% CI: [1.5 – 10.3]; $P = 0.012$). This finding is in agreement with several studies that revealed that elderly patients were more susceptible to DTPs compared to younger adults [28,33,38]. This may be explained by the fact that there is a difference in pharmacokinetics and pharmacodynamics among elderly and pediatric populations [16]. Many medications act differently in older and younger people due to the physiological and pathological changes that accompany aging. According to the American Geriatrics Society (AGS), many medications have different efficacy and safety profiles in younger and older age groups [33]. In addition, studies have demonstrated that elderly patients have comorbidities and usually take complex medication regimens, increasing the risk of DTPs. From the current findings, special attention is warranted for these patient groups by optimizing dosage regimens and following up during medication therapy. This study has revealed that almost 40% of pediatric HF patients had at least one DTP during their hospitalization. Similarly, a multi-center study conducted in four French-speaking countries among pediatric patients in a cardiac and intensive care unit revealed that a significant number of DTPs were recorded during their follow-up [39]. Therefore, there is a need for further studies to be conducted among the pediatric population to mitigate the problem.

In addition, the present study reported that patients who were taking more than 5 medications during their hospital stay were three times more likely to experience DTPs related to cardiovascular agents than those who were taking <5 medications during their hospital stay (AOR: 2.92; 95% CI: [1.5 – 7.6]; $P = 0.029$). This finding is consistent with previously reported studies involving hospitalized HF patients [15,22,28,33,35,40]. Poly-pharmacy has been a significant challenge among CVD patients, but it can be controlled by simplifying the medication regimen by eliminating pharmacologic duplication and regularly reviewing the treatment regimen [40]. Studies have indicated that having clinical pharmacists in the multidisciplinary team in treating CVDs, including HF, would reduce DTPs, improve medication adherence, and increase the treatment satisfaction of HF patients [15,40-42]. For this reason, the clinical pharmacists' intervention could help in addressing this problem and improve the treatment outcome of HF patients.

Our study had several limitations. The study was conducted in a hospital serving referred patients who have severe illnesses and more comorbidity. Hence, the findings may not be representative of the general population. In addition, the DTPs assessed were limited in number and only limited to those associated with cardiovascular agents.

DOI: https://doi.org/10.36922/jctr.23.00137

Table 4. Factors associated with DTPs among hospitalized HF patients at the MRRH

Variable	No DTP, *n* (%)	DTP, *n* (%)	COR (95%CI)	P_{COR}	AOR (95% CI)	P_{AOR}
Age* (years)						
≤18	16 (61.5)	10 (38.5)	1	-	1	-
19 – 59	34 (70.8)	14 (29.2)	0.66 (0.24 – 1.81)	0.416	0.39 (0.12 – 1.2)	0.099
≥60	11 (25)	33 (75)	4.8 (2.1 – 10.6)	0.003#	4.1 (1.5 – 10.3)	0.012#
Gender*						
Male	28 (60.8)	18 (39.2)	1	-	1	-
Female	33 (45.8)	39 (54.2)	0.54 (0.26 – 1.16)	0.113	2.09 (0.86 – 5.06)	0.102
Education						
No formal education	23 (43.4)	30 (56.6)	1.3 (0.37 – 4.57)	0.678	N/A	N/A
Primary	32 (60.3)	21 (39.7)	0.65 (0.18 – 2.31)	0.512	N/A	N/A
Secondary and above	6 (50)	6 (50)	1	-	1	-
Occupation*						
Unemployed	21 (50)	21 (50)	4.0 (0.41 – 12.8)	0.232	2.73 (0.17 – 20.81)	0.477
Self-employed	36 (50.7)	35 (49.3)	3.88 (0.41 – 13.1)	0.235	1.23 (0.10 – 15.47)	0.869
Formally employed	4 (80)	1 (20)	1	-	1	-
Disease status						
New	28 (50)	28 (50)	1	-	1	-
Known	33 (53.2)	29 (46.8)	0.88 (0.43 – 1.8)	0.726	N/A	N/A
Previous hospital admission*						
Yes	44 (56.4)	34 (43.6)	1.75 (0.81 – 3.77)	0.154	0.44 (0.17 – 1.15)	0.093
No	17 (42.5)	23 (57.5)	1	-	1	-
Length of hospital stay* (days)						
≤5	20 (74.1)	7 (25.9)	1	-	1	-
6 – 10	28 (47.4)	31 (52.6)	3.2 (1.2 – 8.6)	0.024#	1.82 (0.54 – 6.09)	0.332
≥11	13 (40.6)	19 (59.4)	4.1 (1.4 – 10.7)	0.012#	2.21 (0.59 –8.34)	0.238
Counseling						
Yes	55 (51.4)	52 (48.6)	1	-	1	-
No	6 (54.5)	5 (45.5)	1.13 (0.33 – 3.9)	0.843	N/A	N/A
Comorbidity*						
Yes	45 (48.3)	48 (51.7)	1.89 (0.76 – 4.72)	0.16	2.52 (0.86 – 5.06)	0.094
No	16 (64)	9 (36)	1	-	1	-
OTC medication use within the past 4 weeks						
Yes	21 (50)	21 (50)	1.11 (0.52 – 2.36)	0.784	N/A	N/A
No	40 (52.6)	36 (47.4)	1	-	1	-
Herbal use within the past 4 weeks*						
Yes	22 (42.3)	30 (57.7)	1.97 (0.94 – 3.11)	0.071	1.52 (0.64 – 3.64)	0.344
No	39 (59.1)	27 (40.9)	1	-	1	-
Alcohol use history						
Yes	18 (54.5)	15 (45.5)	0.85 (0.38 – 1.9)	0.70	N/A	N/A
No	43 (50.6)	42 (49.4)	1	-	1	-
Poly-pharmacy*						
Yes	35 (46.6)	40 (53.4)	1.75 (0.82 – 3.51)	0.151	2.92 (1.5 – 7.6)	0.029#
No	26 (60.5)	17 (39.5)	1	-	1	-
Drug–drug interaction						
Yes	39 (49.4)	40 (50.6)	1.32 (0.62 – 2.8)	0.472	N/A	N/A
No	22 (56.4)	17 (43.6)	1	-	-	-

Note: *Denotes variables with p_{COR}<0.25, selected for further AOR analysis; # $p_{COR/AOR}$ <0.05; N/A denotes AOR (and p_{AOR}) not determined for the respective variable. Abbreviations: COR: Crude odds ratio; AOR: Adjusted odds ratio: CI: Confidence interval: OTC: Over-the-counter; HF: Heart failure; MRRH: Mbarara Regional Referral Hospital; DTPs: Drug therapy problems.

DOI: https://doi.org/10.36922/jctr.23.00137

5. Conclusion

The current findings revealed that almost half of the hospitalized HF patients were experiencing DTPs related to cardiovascular agents. Inappropriate dosing was the most common DTP in this study, followed by failure to initiate recommended medications. Many different independent variables have been identified as determinants for the prevalence of DTP in our study. Among those, elderly patients (i.e., 60 years and older) and patients on five or more concurrent medications were at a significantly higher risk of experiencing DTP related to cardiovascular agents during hospitalization. Based on our findings, the inclusion of a pharmacist in multidisciplinary HF teams should be considered among chronic patients, and patients at a high risk of contracting cardiovascular agent-related DTPs should be given additional follow-ups upon hospital admission.

Acknowledgments

The authors would like to extend their thanks to the study participants for their willingness to participate in this study and all the staff of the internal medicine ward for their cooperation during the data collection process.

Funding

This study was funded by the Inter-University Council for East Africa. The main author Efrata Ashuro Shegena received the award as part of her master's scholarship.

Conflict of Interest

All authors declare that they have no conflict of interest.

Ethical Approval and Consent to Participate

This study was conducted in accordance with the Declaration of Helsinki. Approval to conduct the study was obtained from the Mbarara University Institutional Research Ethics Committee (Reference no.: MUST-2021-185). We obtained site clearance to conduct the study from the MRRH director. Written informed consent was obtained from the patients before participating in the study. The purpose, objectives, benefits, risks, and impact of the study on the total time spent in the hospital were clearly explained to the participants. Participants' confidentiality was maintained during and after data collection.

Consent for Publication

Written informed consent was obtained from all the study participants for publishing the data.

Availability of Data

Data are available from the corresponding author upon reasonable request.

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DOI: https://doi.org/10.36922/jctr.23.00137

Journal of Clinical and Translational Research 2024; 10(3): 201-208

Journal of Clinical and Translational Research

Journal homepage: http://www.jctres.com/en/home

ORIGINAL ARTICLE

Levels of lead in blood and water in occupationally exposed and unexposed population of the Guntur district, Andhra Pradesh: baseline analysis of a prospective cohort study

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ARTICLE INFO

Article history:
Received: November 8, 2023
Accepted: April 13, 2024
Published Online: May 28, 2024

Keywords:
Lead
Water
Occupation
Worker
Pollution

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ABSTRACT

Background: Lead can be ingested, inhaled, or absorbed through the skin, leading to morbidity and mortality.
Aim: This study aimed to estimate and compare the prevalence of high blood lead levels (BLLs) among the adult population with and without occupational lead exposures.
Methods: A baseline survey of a prospective cohort study was conducted in 2022 among 180 adult males and females (20 – 60 years old) in the Guntur district, Andhra Pradesh. The study participants were divided accordingly into three groups: direct occupationally exposed (Group 1); indirect air pollution-exposed (Group 2); and indirect non-occupationally exposed (Group 3). The participants were interviewed using a structured data collection instrument. Blood and water lead levels were estimated using a graphite furnace atomic absorption spectrophotometer. We defined statistical significance as $P < 0.05$.
Result: Among the studied participants, 65.56% were less than 40 years of age and 74.44% were males. The BLLs ranged from 2.15 µg/dL to 19.03 µg/dL. The mean BLLs were 8.50 ± 2.36, 7.34 ± 3.02, and 5.65 ± 2.91 µg/dL for Groups 1, 2, and 3, respectively. The lead content in samples of 20 L-canned water in each group was more than 10 µg/L. On adjustment in multivariate analysis, the male gender and direct occupational exposure are significant risk factors for high BLLs (i.e., ≥5 µg/dL).
Conclusion: Both occupationally exposed and unexposed groups in the study had higher mean BLLs than recommended. The mean BLL in the occupationally exposed group was significantly higher compared to the general population. Higher lead content in drinking water may expose individuals to lead-related symptoms.
Relevance for Patients: High BLLs can have significant negative health effects on the human body. Lead is particularly harmful to the central nervous system and cardiovascular system.

1. Introduction

Lead occurs naturally in the Earth's crust and poses significant toxicity to humans when ingested, inhaled, or absorbed through the skin [1]. It persists in various environmental mediums, such as soil, air, drinking water, and homes, where it accumulates and does not degrade [1]. High levels of lead exposure can have adverse effects on adults, such as inducing coma, convulsions, and death [1]. Reports have identified six primary

DOI: https://doi.org/10.36922/jctr.23.00130

sources that significantly contribute to lead exposure: gasoline additives; food can solder; lead-based paints; ceramic glazes; drinking water systems; and cosmetic and folk remedies [2]. Other significant exposures include inadequately controlled industrial emissions from lead smelters and battery recycling plants, contaminating both the environment and people in the vicinity [2]. The highest level of environmental contamination is found to be associated with uncontrolled recycling operations, with the most highly exposed adults being those who work with lead [3].

In India and most developing countries, the main source of lead pollution was previously automobile exhaust. With the use of unleaded petrol, lead pollution due to automobile exhaust has drastically dropped [4]. Approximately 143,000 people die annually from lead poisoning, accounting for 0.6% of the global disease burden [5], and Southeast Asia accounts for over half of the global burden of lead-related illness. Greater blood lead levels (BLL) are linked to increased all-cause mortality in both men and women with cardiovascular diseases. Although the World Health Organization (WHO) has set a standard BLL of 5 µg/dL for adults [1], the Environmental Health Committee of the Council of State and Territorial Epidemiologists (CSTE) indicated that a blood lead reference value (BLRV) >3.5 µg/dL is considered high. BLRV is used to identify patients with the highest BLL in the population but is not indicative of a toxicity threshold [6]. Adult lead toxicity is typically considered at mean BLL ≥10 µg/dL, but there is evidence linking long-term risks to chronic lead exposure below 10 µg/dL [7]. Other studies indicate a correlation between higher BLLs and increased cardiovascular mortality in adults [8]. Lead is a strong inhibitor of *δ*-aminolevulinic acid dehydratase, affecting the spleen and hematopoietic system [9].

According to evidence on the long-term effects of low-level lead exposures and the prevalence of lower levels in the population, the United States (US) Department of Health and Human Services advises reducing BLLs among all individuals to <10 µg/dL [7,10,11]. It is widely recognized that the lead exposure standard set by the US Occupational Health and Safety Administration is outdated and does not provide adequate protection against lead poisoning [10,12]. This standard permits workers to continue working in lead-exposed environments with BLLs of up to 40 µg/dL.

Developed countries, such as the US, United Kingdom, and Germany, have implemented aggressive measures to address lead poisoning while developing countries present slower and more sporadic actions. Within the past decade, there have been numerous reports of lead poisoning in humans, particularly from developing countries faced with environmental and occupational lead exposure [4]. The present study was conducted to estimate and compare BLLs in the adult population with and without occupational lead exposure.

2. Methods

2.1. Study design

A baseline survey of the prospective cohort study was conducted in the Guntur district, Andhra Pradesh, India, which was approximately 30 km from the All India Institute of Medical Sciences (AIIMS), Mangalagiri. The study was conducted in 2022 among adult males and females from 20 to 60 years of age. The study participants were divided accordingly into three groups:

(i) Group 1: Direct occupationally exposed individuals, such as workers in lead battery manufacturing, construction workers, demolition workers, gas station attendants, lead smelters, smolderers, and painters.
(ii) Group 2: Indirect air pollution-exposed individuals, such as traffic police, police, truck drivers, bus drivers, auto drivers, and petrol bunk workers.
(iii) Group 3: Indirect non-occupationally exposed individuals, such as indoor officer workers, teachers, primary health-care workers, and housewives.

Individuals were eligible for participation in Groups 1 and 2 after working in the same occupation for at least 6 months or in Group 3 after residing in the area or working in the same occupation for the past 6 months. Individuals with symptoms suggestive of critical illness, diabetes mellitus, hypertension, recently underwent surgery, and those who denied consent were excluded from the study.

The sample size was calculated using the sample formula required per group:

$$n = \left(\sigma_1^2 + \sigma_2^2\right) \times \frac{[Z_{1-@/2} + Z_{1-\beta}]^2}{(M_1 - M_2)^2} \quad \text{(I)}$$

Where σ_1 denotes the standard deviation (*SD*) of the outcome variable in Group 1, σ_2 denotes the *SD* of the outcome variable in Group 2, $Z_{1-@/2}$ and $Z_{1-\beta}$ denote the probability of two types of errors at 1.96 and 1.282, respectively, and $M_1 - M_2$ denotes the mean difference between groups. The means of continuous variables were compared using a *t*-test. We utilized the findings from two previous studies as Groups 1 and 2 to determine the sample size. Group 1 included workers handling raw material in a battery factory in Nellore, Andhra Pradesh, with a mean ± 2*SD* BLL (µg/dL) of 26.2 ± 2.142 in 2016 – 2017 [3]. Group 2 included non-occupationally lead-exposed healthy school teachers from various public and government sectors, representing various nodal areas of Jodhpur with a mean ± 2*SD* BLL (µg/dL) of 6.89 ± 9.5 [13]. Utilizing a 95% confidence interval (CI) and 90% power, we calculated the required sample size to detect a 3.0 µg/dL difference in BLLs in any two groups to be 60. Hence, the total sample studied was 180, excluding nonresponse and attrition.

After ascertaining the eligibility of the participant, detailed information about the study was provided through the Participant Information Sheet and consent document. Both these documents were in Telugu, and any difficult words were explained with the assistance of a local interpreter. Data collection was conducted once written consent was accorded. The project was approved by the ethical committee of the AIIMS Institute (AIIMS/MG/IEC/2022-2023/135).

Investigators were trained in data collection, blood collection, and transportation. A pilot survey was conducted and corrective

DOI: https://doi.org/10.36922/jctr.23.00130

measures were taken. A structured data collection instrument, comprising information about sociodemographic details (e.g., age, smoking status, alcohol ingestion, and duration of occupational exposure) and clinical details, was developed. This instrument was pretested, suitably modified, and subsequently implemented. Basic sociodemographic information (e.g., family size, age, education, occupation, gender of members of the family, and occupational years) was collected to study the correlation of these factors to the risk of high BLL.

In this study, BLL was considered the outcome variable, while the exposure variables include the participants' occupation, age, education, smoking status, and alcohol intake, among others.

2.2. Blood sample collection

Blood samples (3 mL) were collected under sterile conditions using BD Vacutainer® Eclipse™ blood collection needles (368608; BD, USA) into BD Vacutainer® EDTA tubes (367861; BD, USA) containing EDTA K2 anticoagulant for BLL measurements. The blood samples were transported to the AIIMS Mangalagiri Biochemistry Laboratory, while maintaining a cold chain (i.e., in cold boxes with temperature monitors). At the laboratory, the samples were processed accordingly. In a 100 mL volumetric flask, 5 mL of 10% Triton X-100, 2 mL of NH_4PO_4, and four drops of 70% HNO_3 were mixed and diluted to volume with deionized water to form the matrix modifier. To prepare a multipoint calibration curve, 0.1, 5, 10, 30, and 60 µg/dL working standard lead-containing solutions were prepared in 1% HNO_3. The final standard solutions were prepared by mixing 100 µL of each of the working standard solutions with 900 µL of matrix modifier in autosampler vessels to produce 0.1, 0.5, 1, 3, and 6 µg/dL, respectively. These standard solutions were set aside until the bubbles dissipated. The samples were then prepared by mixing 100 µL of whole blood (with anticoagulant) with a 900 µL matrix modifier. BLLs were estimated using a graphite furnace atomic absorption spectrophotometer. The trace element blood collection tubes used here refer to BD Vacutainer® specialty tubes (368381; BD, USA). The BLL measurement method has been validated with an estimated detection limit of <1 µg/dL and requires only a small sample size. The measurement method also has a multi-element capacity with little interference.

Information about the study was shared with the communities through field health workers, schools, Anganwadi, and social media. Any eligible participants (for either one of the three groups) visiting the AIIMS hospital or the Centre for Rural Health AIIMS Nutakki were enrolled using purposively sampling. Furthermore, independent camps were conducted at peripheral centers. The investigator introduced himself/herself to the participant before the start of the interview. Individuals were given patient information sheets. Thereafter, the research team explained the study, its objectives, procedure, and the rights of the participants. If the individuals agreed to participate in the study after going through the information sheet, written consent was obtained. A unique code was assigned to each participant. The participants were interviewed according to the interview schedule, and blood sampling was conducted after the interview. The laboratory technicians were kept blinded, and all blood testing reports were shared with the participants.

The four water sources were evaluated in each group of participants. The first sample was the first water from the tap in any randomly selected participants' houses within the study area. The second water sample was from a 20-L packed plastic can of water available in the vicinity of the above house. The third water sample was from the water purifier in any of the randomly selected participants' houses. The fourth water sample was from the tap at the nearest health-care facility, school, or office from the selected participants' house.

2.3. Data analysis

Data were entered in Microsoft Excel and analyzed by using IBM SPSS Statistics Base version 28.0. Lead exposures at baseline were categorized into three groups. The continuous variables in the study (i.e., BLLs) were summarized as mean ± *SD*. Normality was assessed using the Kolmogorov–Smirnov test. The categorical variables, including age, gender, and occupational exposure, were presented as frequency or percentage.

Bivariate analysis of categorical parameters, i.e., comparison of BLLs, was performed using the Chi-square (χ^2) test. The mean BLLs of the three groups were compared using analysis of variance with *post hoc* analysis. Multivariate logistic regression was performed to distinguish the exposure variables according to BLL < or ≥5 µg/dL. Statistical significance was defined as $P < 0.05$.

3. Results

Approximately 65.56% of participants were less than 40 years old, with a mean age of 35.65 ± 9.21 years; 74.44% of participants were males; 68.33% of participants were are at least 10th class (i.e., more educated); 47.22% of participants belonged to the upper middle socioeconomic status (Table 1). The median (interquartile range [IQR]) of family and individual incomes were INR 20000 (15000) and INR 15000 (8500), respectively.

Table 2 reports that 17.78% of participants were smokers and 3.8% used smokeless tobacco. Overall, 37.78% of participants reported that their house was within a 1 km radius of the highway or traffic zone. Approximately 50.56% of participants were using 20-L canned water for drinking.

Table 3 reports that 36.7% of participants were painters and 30.0% were construction workers in Group 1; 50.0% were traffic police and 33.3% were auto drivers in Group 2; and 41.7% were primary health-care workers (accredited social health activist [ASHA] and Anganwadi) and 40.0% were office workers in Group 3. The median (IQR) working hours in a typical day was 8 (2) h. The median (IQR) number of years in the present occupation of study participants was 10 (11.7) years.

Figure 1 displays the violin plot of the distribution of participants for BLLs and the group studied. Overall, 56 (93.3%) participants in Group 1, 46 (76.6%) participants in Group 2, and 28 (46.6%) participants in Group 3 had BLLs >5 µg/dL of blood. The BLL ranged from 2.15 µg/dL to 19.03 µg/dL.

DOI: https://doi.org/10.36922/jctr.23.00130

Table 1. Distribution of study participants by their sociodemographic factors (n=180)

Sociodemographic factor	Number of participants, *n* (%)				χ^2	*P*
	Cat 1	Cat 2	Cat 3	Total		
Age (years)					33.12	<0.001*
<40	27 (45.0)	35 (58.3)	56 (93.3)	118 (65.56)		
≥40	33 (55.0)	25 (41.7)	4 (6.7)	62 (34.44)		
Gender					108.16	<0.001*
Female	0 (0.0)	2 (3.3)	44 (73.3)	46 (25.56)		
Male	60 (100.0)	58 (96.7)	16 (26.7)	134 (74.44)		
Education (class)					17.09	<0.001*
<10	29 (48.3)	20 (33.3)	8 (13.3)	57 (31.67)		
≥10	31 (51.7)	40 (66.7)	52 (86.7)	123 (68.33)		
Migrant					5.92	0.05
Yes	8 (13.3)	19 (31.7)	16 (26.7)	43 (23.89)		
Living with family					0.7	0.7
Yes	56 (93.3)	57 (95.0)	58 (96.7)	171 (95.00)		
Socioeconomic status						
Upper	4 (6.7)	24 (40.0)	17 (28.3)	45 (25.00)	23.21	0.001*
Upper middle	31 (51.7)	23 (38.3)	31 (51.7)	85 (47.22)		
Lower middle	20 (33.3)	12 (20.0)	11 (18.3)	43 (23.89)		
Lower	5 (8.3)	1 (1.7)	1 (1.7)	7 (3.89)		

Note: Socioeconomic status is determined using the BG Prasad scale; *p < 0.05 denotes statistical significance.
Abbreviation: Cat: Category.

Table 2. Distribution of study participants by their behavior and residential factors (n=180)

Behavior/residential factor	Number of participants, *n* (%)				χ^2	*P*
	Cat 1	Cat 2	Cat 3	Total		
Smoking					21.51	<0.001*
Yes	13 (21.7)	19 (31.7)	0 (0.0)	32 (17.78)		
Smokeless chewable tobacco					14.56	0.001*
Yes	7 (11.7)	0 (0.0)	0 (0.0)	7 (3.89)		
Alcohol use					34.71	<0.001*
Yes	22 (36.7)	27 (45.0)	0 (0.0)	49 (27.22)		
Residence within 1 km of highway/traffic zone					17.92	0.001*
Maybe	14 (23.3)	16 (26.7)	18 (30.0)	48 (26.67)		
No	33 (55.0)	13 (21.7)	18 (30.0)	64 (35.56)		
Yes	13 (21.7)	31 (51.7)	24 (40.0)	68 (37.78)		
Drinking water					20.42	0.002*
Household filtered/RO water	1 (1.7)	8 (13.3)	16 (26.7)	25 (13.89)		
20 L-canned water	39 (65.0)	30 (50.0)	22 (36.7)	91 (50.56)		
Municipality	17 (28.3)	18 (30.0)	15 (25.0)	50 (27.78)		
Others	3 (5.0)	4 (6.7)	7 (11.7)	14 (7.78)		
Personal protective					12.33	0.015*
No/not applicable	45 (75.0)	47 (78.3)	40 (69.0)	132 (73.33)		
Sometimes	8 (13.3)	0 (0.0)	3 (5.2)	11 (6.11)		
Yes	7 (11.7)	13 (21.7)	15 (25.9)	35 (19.44)		

Note: *P < 0.05 denotes statistical significance.
Abbreviation: Cat: Category; RO: Reverse osmosis.

The mean blood levels of lead were 8.50 ± 2.36, 7.34 ± 3.02, and 5.65 ± 2.91 µg/dL for Groups 1, 2, and 3, respectively. The BLLs among the three groups of participants were statistically significant (P < 0.001). The mean BLL was highest in Group 1, followed by Groups 2 and 3. There was also a statistically significant difference in the mean BLLs of Group 1 vs. Group 2 (P = 0.03), Group 2 vs. Group 3 (P = 0.007), and Group 1 vs. Group 3 (P < 0.001).

Table 4 reports the lead content in water samples among the three studied groups. The lead content in samples of 20 L-canned

DOI: https://doi.org/10.36922/jctr.23.00130

water in each group was more than 10 µg/L. In Group 2, the lead levels were 29.34 µg/L in a sample of household filter water and 18.06 µg/L in a sample of facility-based water. In Group 3, the lead levels were 16.43 µg/L in a sample of municipality water.

Bivariate analysis revealed that age ≥40 years, male gender, direct occupational exposure, education <10th class, lower socioeconomic status, use of alcohol, and drinking from 20 L-canned water are significant risk factors for high BLLs, i.e., ≥5 µg/dL. On adjustment in multivariate logistic regression, male gender and direct occupational exposure are significant risk factors for high BLLs, i.e., ≥5 µg/dL (Table 5).

Table 3. Distribution of participants in the three groups by occupation

Occupation	Number of participants, *n* (%)			
	Group 1	Group 2	Group 3	Total
Carpenter, or welding worker	15 (25.00)			15 (8.30)
Car mechanics	5 (8.30)			5 (2.80)
Painters	22 (36.70)			22 (12.20)
Construction worker	18 (30.00)			18 (10.00)
Petrol bunkers		8 (13.33)		8 (4.44)
Auto drivers		20 (33.30)		20 (11.10)
Traffic police		30 (50.00)		30 (16.70)
Driver		2 (3.30)		2 (1.10)
Office workers			24 (40.00)	28 (15.60)
ASHA and Anganwadi worker			25 (41.70)	25 (13.90)
Teachers			5 (8.30)	5 (2.80)
Student			2 (3.30)	2 (1.10)
Manual labor			2 (3.30)	2 (1.10)
Housewife			1 (1.70)	1 (60.00)
Private job			1 (1.70)	1 (1.70)
Total	60 (100.00)	60 (100.00)	60 (100.00)	180 (100.00)

Abbreviation: ASHA: Accredited social health activist.

Table 4. Lead content in water samples among the three studied groups (*n*=12)

Sample type	Water lead content (µg/L)		
	Group 1	Group 2	Group 3
20 L-canned water	16.38	12.23	65.11
Household filter water	7.28	29.34	7.56
Facility-based water	4.79	18.06	7.71
Municipality water	7.1	7.5	16.43

4. Discussion

The present study was conducted to estimate and compare BLLs among the adult population with and without occupational lead exposure. The present study was the first of its kind for its community-based BLL estimation among three different exposed groups in Andhra Pradesh, India, namely direct occupational exposure, indirect air pollution exposure, and indirect non-occupational exposure to lead. This study found that the majority of participants had high BLLs. The WHO has established a reference value of 5 µg/dL BLL as the threshold at which public health action is recommended [14]. In the present study, mean BLLs in all three groups were higher than the reference value. In India, the Bureau of Indian Standards set a maximum permissible limit of 10 µg/L for lead in drinking water [15]. The lead content in all four samples of 20 L-canned water and one reverse osmosis (RO) plant was more than permissible.

Occupational lead exposure may occur in various labor-based fields, such as construction, painting, smelting, and others. Therefore, the BLL of these workers is much higher than in the general population. In the air, lead particles can be inhaled by individuals and enter their bloodstream. The WHO has established a guideline value for lead in outdoor air of 0.5 µg/m^3 [16]. Despite the use of unleaded fuels in some parts of India, lead levels in outdoor air still exceed this guideline [17].

In the present study, the not occupationally exposed group mainly consisted of primary health-care workers and office-based workers. While not being exposed to occupational lead or air pollution zones, the mean BLL in this group was also more than 5 µg/dL. One of the primary reasons for the high BLL in this group could be the use of 20 L-canned water for drinking purposes. The finding suggests that the purification techniques for these water plants are suboptimal. RO plants should be equipped with updated technology for testing heavy metals. Although lead can enter the water supply from a variety

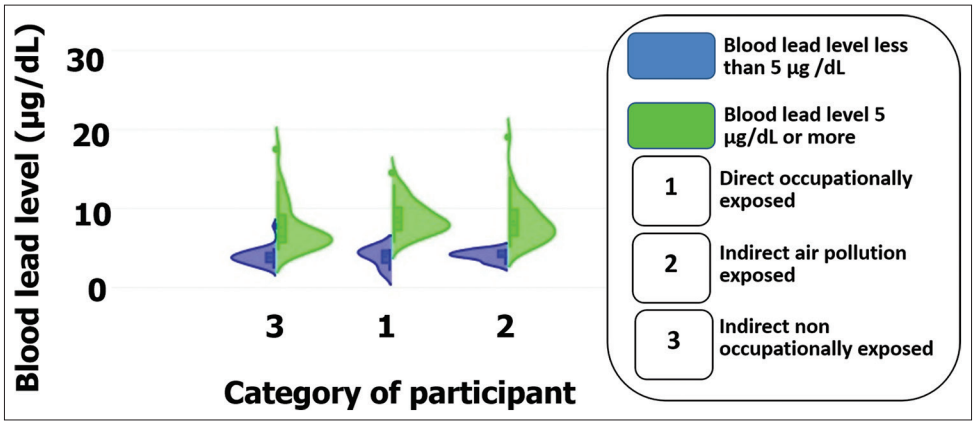

Figure 1. Distribution of study participants by blood lead levels.

DOI: https://doi.org/10.36922/jctr.23.00130

Table 5. Logistic regression analysis of risk factors of blood lead level (BLL) ≥ 5 μg/dL

Risk factor	Number of participants, n (%)			Bivariate logistic regression		Multivariate logistic regression	
	BLL <5 μg/dL	BLL ≥5 μg/dL	Total	COR (95% CI)	*P*	AOR (95% CI)	*P*
Age (years)							
<40^	39 (33.1)	79 (66.9)	118 (65.6)	0.43 (0.21 – 0.93)	0.029*	1.03 (0.38 – 2.76)	0.947
≥40	11 (17.7)	51 (82.3)	62 (34.4)				
Gender							
Female^	25 (54.3)	21 (45.7)	46 (25.6)	0.19 (0.9 – 0.39)	< 0.001*	0.32 (0.12 – 0.85)	0.023*
Male	25 (18.7)	109 (81.3)	134 (74.4)				
Occupation (exposure)							
Direct^	4 (6.7)	56 (93.3)	60 (33.3)	8.70 (2.96 – 26.6)	< 0.001*	4.15 (1.06 – 16.26)	0.04*
Indirect	46 (38.3)	74 (61.7)	120 (66.7)				
Education (class)							
<10^	10 (17.5)	47 (82.5)	57 (31.7)	2.26 (1.04 – 4.94)	0.037*	1.18 (0.45 – 3.08)	0.735
≥10	40 (32.5)	83 (67.5)	123 (68.3)				
Socioeconomic status							
Upper or upper middle^	25 (55.6)	20 (44.4)	45 (25.0)	0.35 (0.17 – 0.73)	0.004*	0.47 (0.19 – 1.16)	0.102
Middle or lower-middle	105 (77.8)	30 (22.2)	135 (75.0)				
Smoking							
Yes^	5 (15.6)	27 (84.4)	32 (17.8)	2.35 (0.85 – 6.52)	0.09	0.85 (0.21 – 3.35)	0.823
No	45 (30.4)	103 (69.6)	148 (82.2)				
Smokeless chewable tobacco							
Yes^	1 (14.3)	6 (85.7)	7 (3.9)	2.37 (0.27 – 20.21)	0.41	N/A	N/A
No	49 (28.3)	124 (71.7)	173 (96.1)				
Alcohol							
Yes^	6 (12.2)	43 (87.8)	49 (27.2)	3.62 (1.43 – 9.16)	0.004*	2.27 (0.67 – 7.62)	0.184
No	44 (33.6)	87 (66.4)	131 (72.8)				
Highway within a 1 km radius of residence							
Yes^	53 (77.9)	15 (22.1)	68 (37.8)	1.61 (0.79 – 3.23)	1.78	N/A	N/A
No	77 (68.8)	35 (31.2)	112 (62.2)				
Drinking water							
20 L-canned water^	16 (17.6)	75 (82.4)	91 (50.6)	2.89 (1.45 – 5.76)	0.002*	2.16 (0.98 – 4.73)	0.05
Others	34 (38.2)	55 (61.8)	89 (49.4)				

Note: *P<0.05 denotes statistical significance; variables with P>0.2 were not included in multivariate analysis (denoted by N/A); ^denotes the reference value.
Abbreviations: AOR: Adjusted odds ratio; CI: Confidence interval; COR: Crude odds ratio; N/A: Not available.

of sources, including old lead pipes, plumbing fixtures, lead solder used in plumbing, and lead-containing valves or fittings, the municipality water supply was found to have lead within permissible limits.

The present study results were comparable to those of recent studies: a meta-analysis of 31 studies involving the Indian population, i.e., 5472 people across nine states, reported a mean BLL of 7.52 μg/dL (95% CI: 5.28 – 9.76) in non-occupationally exposed adults [18]; a cross-sectional study of 32 male painters in Iran in 2021 reported a mean BLL of 8.1 ± 4.93 μg/dL [19]; a cross-sectional study among 254 workers aged 20 – 60 years old, at a battery factory in Nellore, Andhra Pradesh, in 2016 – 17 reported a mean BLL of 25.26 ± 2.1 μg/dL[3]; and a study from Turkey in 2001 among 99 traffic policemen reported a mean BLL of 9.4 ± 1.6 μg/L and 8.7 ± 1.7 μg/L for policemen working outdoors and indoors, respectively [20]. Our results were also lower compared to a study from China [21]. This could be due to workers in these industries being exposed to higher lead through inhalation of dust or fumes, ingestion of contaminated food or water, or in direct contact with lead-containing materials compared to the general population.

The present study reported mean BLLs higher than the recommended value in all three studied groups. Among these, the direct occupationally exposed group had the highest mean BLL, while lay people from the community had the lowest mean BLL. Occupation exposure and high water lead content are probably the causative factors for higher BLLs in this population. High BLLs can have significant negative health effects on the human body. Lead is particularly harmful to the central nervous system and cardiovascular system and can accumulate in the kidneys over time, leading to kidney damage. Lead can also interfere with the development and maintenance of healthy bones. However, there were some limitations of this study. While participants reported various symptoms, such as joint pains, headaches, abdominal pain, and muscle pain/fatigue, establishing a direct causal relationship with BLL alone is challenging per se. Detailed clinical and biological investigations are required to rule out

DOI: https://doi.org/10.36922/jctr.23.00130

other possible causes of these symptoms. In addition, the number of water samples collected was limited, and the study did not measure lead content in air samples. These limitations may affect the scope of the study to establish a clear causal relationship between the different factors and high BLLs among the study population.

5. Conclusion

Both occupationally exposed and unexposed groups in the study had higher mean BLL than recommended. The mean BLL in the occupationally exposed group was significantly higher compared to the general population. Higher lead content in drinking water exposes individuals to lead-related symptoms. Future estimations must be informed by larger and population-wide BLL research. Governments and public health organizations can mitigate lead exposure from water contamination by implementing measures such as mandatory labeling and periodic monitoring for 20 L-canned water available in local markets for drinking purposes.

Acknowledgments

We are thankful to the traffic police in Guntur, the Auto Union in Mangalagiri, auxiliary nurse midwives (ANMs), ASHAs, staff nurses, and Anganwadi workers for all their support for the study. We also thank Dr. Sai Subhakar Desu for his support in the data collection, Siva Santosh Kumar Pentapati, Mounika Kollimarla and Ms. Pulla Sirisha for supporting the study. We also thank all the staff of the Centre for Rural Health AIIMS (CRHA), PHC Nutakkhi for their valuable support in the conduct of the study.

Funding

This study was funded by the Intramural Funds (2021 – 2022), AIIMS Mangalagiri.

Conflict of Interest

The authors declare no conflict of interest.

Ethics Approval and Consent to Participate

The project was approved by the ethical committee of the AIIMS Institute [AIIMS/MG/IEC/2022-2023/135]. Informed written consent was obtained from the participants for releasing their data without any personnel identifier before enrollment in the study.

Consent for Publication

Informed written consent was obtained from the participants for releasing their data without any personnel identifier before enrollment in the study.

Availability of Data

Data are available from the corresponding author upon reasonable request.

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DOI: https://doi.org/10.36922/jctr.23.00130

Journal of Clinical and Translational Research 2024; 10(3): 209-211

Journal of Clinical and Translational Research

Journal homepage: http://www.jctres.com/en/home

LETTER

Genetic and environmental influences on vaccine hesitancy

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ARTICLE INFO

Article history:
Received: January 11, 2024
Accepted: March 27, 2024
Published Online: June 5, 2024

Keywords:
COVID-19
Vaccine hesitancy
Genetics
Environment
Twins

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1. Introduction

Polarizing attitudes toward the COVID-19 vaccine continue to impede public health efforts to control the spread of the SARS-Cov-2 virus. Approximately 80% of the US population report having been vaccinated at least once, but refusal rates are as high as 30% in some states. Despite the recommendations from the Centers for Disease Control and Prevention (CDC), only 22% of adults have received an updated second dose of the vaccine [1].

The present study was conducted to examine the role of genetic and environmental factors in vaccine hesitancy. Early twin studies of attitudes suggest that both genetic and environmental factors contribute to differences in political and social behavior [2,3]. For example, political affiliation and conservatism, attitudes that have been associated with vaccine resistance, have a significant genetic influence. Social-environmental factors have been strongly implicated in vaccine hesitancy, but the extent to which genetic factors contribute remains uncertain [4]. Given the rising rates of COVID-19 and the emergence of new COVID variants, we utilized a twin design to assess the genetic and environmental influences on vaccine acceptance (and hesitancy).

2. Methods

2.1. Subjects

Between June 2020 and October 2021, information on acute COVID-19 and vaccine acceptance was collected on monozygotic (MZ) and dizygotic (DZ) adult twins, recruited from the Mid-Atlantic Twin Registry (MATR). Twins were eligible to participate if both twins were registered in the MATR and if at least one twin had been seen within the Virginia Commonwealth University (VCU) health system. Self-report data were collected using an online research platform developed by Vibrent (United States of America).

2.2. Assessment

Twins were asked: “Since January 2020, have you been sick for more than 1 day with an illness related to COVID-19 symptoms? Did you receive the COVID-19 vaccine in the past year? If not, when a COVID-19 vaccine is available, how likely are you to want to get the vaccine? If not, what factors make you less likely to get the vaccine?”. A validated series of questions on twin similarity was used to classify the twins as MZ or DZ [5]. The study was approved by the VCU Institutional Review Board (IRB) (#HM200021382).

DOI: http://doi.org/10.36922/jctr.24.00002

Table 1. Additive genetic, shared environmental, and non-shared environmental components of variance for vaccine hesitancy

Model	Genes	Shared environment	Non-shared environment	−2 ln (L)	*Df*	χ^2 diff	*P*
ACE	0.0	0.77	0.23	489.77	3	-	-
E	0.0	0.0	1.00	548.65	5	58.88	<0.0001
CE*	0.0	0.77	0.23	489.77	4	0	ns
AE	0.35	0.0	0.65	495.88	4	6.11	<0.001

Note: *Best-fitting model.
Abbreviations: −2 ln (L): Goodness of fit; A: Genes; C: Shared environment; E: Non-shared environment; *Df*: Degrees of freedom; ns: Non-significant; χ^2 diff: Chi-square difference.

2.3. Data analysis

The comparison in similarity of MZ versus DZ twins is the foundation for estimating the contribution of genetic, shared environmental, and non-shared environmental factors to vaccine hesitancy [6]. Additive genetic effects reflect the average effect of individual alleles and genetic loci of a trait. Because MZ twins (on average) share 100% of their genes and DZ twins 50% of their genes in common, a higher MZ correlation to DZ correlation suggests that genetic factors are influencing the trait. Common environmental effects describe influences which make family members more alike compared to random pairs of individuals, such as peers, family, and the wider community.This shared environment is reflected in a DZ correlation greater than one-half the MZ correlation. Unique environmental factors (including error of measurement) are those variables that affect only one MZ twin of the pair and create differences in MZ twins despite their identical genotypes. Tetrachoric correlations for vaccine acceptance in the MZ and DZ twins were estimated using SAS software [7]. Genetic and environmental models, controlling for age and sex, were fitted to the twin data using the statistical program OpenMx [8].

3. Results and Discussion

Female twins, particularly MZ twins, were more highly represented than male twins. The age range of the twins was 18.2 – 72.4 with a median age of 35.1 for the MZ twins and 34.8 for the DZ twins.

Nearly half of the twins (47%) indicated that they had acute COVID-19 symptoms since 2020 (n = 540). By October 2021, 90% of the sample indicated that they were vaccinated (n = 1035). From a potential sample of 3586 twins, 1793 were successfully contacted via email or phone. Out of these, 1150 individual twins, comprising 325 MZ and 115 DZ twin pairs, provided their data.

Of the 115 twins that were not vaccinated: (i) 60% indicated "a lack of trust" was the reason for not getting the vaccine; (ii) 10 – 20% said: "It will not help," "Vaccination is worse than being ill," "It is just a virus/not fatal/not necessary," "It depends on the risks/adverse events," "I am not in a risk group with underlying conditions," and/or "I need more information first;" and less than 10% said: "I will not get/am never sick," "I never get vaccinated," "I do not want to pay for it," and/or "My region is not a high-risk area."

The tetrachoric correlations for vaccine acceptance indicate a high degree of similarity in the MZ and DZ twins (0.78 vs. 0.81, respectively), suggesting that genes have little effect on one's willingness to be vaccinated. Table 1 displays the results of the model fitting, inclusive of alternative models, their goodness of fit (−2 ln[L]), and the Chi-square (χ^2) difference between them. The standardized genetic and environmental components of variance are reported for each model. The full model comprised additive genetic factors (A), shared environmental factors (C), and non-shared environmental factors (E) and was tested against three alternative models: (i) A model with unique environmental factors alone (E); (ii) a model without a genetic influence (only C and E); and (iii) a model without the shared environment (only A and E).

The full three-parameter model provided a good fit to the data (−2 ln[L] = 489.77). Eliminating the genetic parameter (A) did not affect the fit of the model (−2 ln[L] = 489.77; 1 degree of freedom [Df]), whereas eliminating the shared environment (C) resulted in a significantly worse fit (−2 ln[L] = 495.88; χ^2 difference = 6.11; 1 Df; $P < 0.001$). For the best-fitting CE model, 77% of the variation in vaccine hesitancy is accounted for by environmental factors shared by the twins.

4. Conclusion

This study provides strong empirical support for the role of the environment in vaccine acceptance. In contrast to studies of other social–political attitudes, genetic factors do not play a role. The overwhelming information from the media and government agencies about getting vaccinated is the most likely explanation for these findings. A lack of trust was by far the most important reason for vaccine hesitancy. The results underscore the need for bold new strategies to expedite the acceptance of the COVID-19 vaccine and other vaccines that offer protection from viral outbreaks in the future.

Acknowledgments

The project was facilitated by the Mid-Atlantic Twin Registry administered by the Cohort the Registry Administration (CARA) Core, a Virginia Commonwealth University shared resource. We would like to extend our gratitude to all of the MATR twins who took part in this study.

Funding

This study was funded by NIH UL1TR002649.

Conflict of Interest

The authors declare no conflicting interest.

Ethics Approval and Consent to Participate

Informed consent was obtained before the subjects participated in the study through the virtual data platform. The study was approved by the VCU IRB (HM200021382).

DOI: http://doi.org/10.36922/jctr.24.00002

Consent for Publication

The consent includes the possibility of data sharing. Requests for data are facilitated by the Mid-Atlantic Registry (MATR) according to IRB-approved Data Transfer and User Agreements.

Availability of Data

Data are available from the corresponding author on reasonable request.

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DOI: http://doi.org/10.36922/jctr.24.00002

Journal of Clinical and Translational Research 2024; 10(3): 212-218

Journal of Clinical and Translational Research

Journal homepage: http://www.jctres.com/en/home

ORIGINAL ARTICLE

Development and validation of an *ex vivo* porcine model of functional tricuspid regurgitation

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ARTICLE INFO

Article history:
Received: January 28, 2024
Accepted: March 21, 2024
Published Online: June 5, 2024

Keywords:
Ex vivo
Functional tricuspid regurgitation
Porcine
Translational

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ABSTRACT

Background and Aim: *Ex vivo* models of functional tricuspid regurgitation (FTR) are needed for pre-clinical testing of novel surgical and interventional repair strategies, but current options are costly or have not been formally validated. The objective of this research was to create and validate an *ex vivo* model to test novel repair methods for FTR.

Methods: In explanted porcine hearts, the right atrium was excised to visualize the tricuspid valve. The pulmonary artery and aorta were clamped and cannulated, the coronary arteries ligated, and the right and left ventricles statically pressurized with air to 30 mmHg and 120 mmHg, respectively. FTR was induced by increasing right ventricular pressure to 80 mmHg for 3 h, which resulted in progressive tricuspid annular enlargement, right ventricular dilation, papillary muscle displacement, and central tricuspid malcoaptation. A structured light scanner was used to image the 3D topography of the tricuspid valve in both the native and FTR state, and images were exported into scan-to-computer-aided design software, which allowed for high-resolution 3D computational reconstruction. Relevant geometric measurements were sampled including annular circumference and area, major and minor axis diameter, and tenting height, angle, and area. Geometric measurements from the *ex vivo* model were compared to clinical transthoracic echocardiographic (TTE) measurements using two-sample *t*-tests.

Results: A total of 12 porcine hearts were included in the study. Annular measurements of the native valve were comparable to published TTE data, except for the minor axis diameter, which was shorter in the *ex vivo* model (2.5 vs. 3.1 cm, $P = 0.007$). Induction of FTR in the *ex vivo* model resulted in annular enlargement (FTR vs. native: circumference 13.7 vs.11.8 cm, $P = 0.012$; area 14 vs. 11 cm^2, $P = 0.011$). *Ex vivo* leaflet measurements in both the native and FTR model differed from published TTE data, but demonstrated comparable directional changes between the native and regurgitant states, including increased tenting height, area, and volume.

Conclusion: The *ex vivo* pneumatically-pressurized porcine model closely recapitulates the geometry of both the native and regurgitant tricuspid valve complex in humans and holds promise for testing novel FTR repair strategies.

Relevance for Patients: Currently available interventions for the tricuspid valve have a risk of permanent conduction abnormalities and are insufficient in addressing tricuspid disease for a subset of patients. This *ex vivo* model provides a platform for testing of novel interventions that address the deficiencies of current tricuspid therapies.

1. Introduction

Functional tricuspid regurgitation (FTR) is the most prevalent tricuspid valve abnormality and refers to regurgitation that occurs in the absence of leaflet abnormalities [1]. The most common repair strategy for FTR is tricuspid annuloplasty, but this strategy carries a risk of conduction abnormalities requiring permanent pacemaker implantation and is

DOI: https://doi.org/10.36922/jctr.24.00003

not uniformly effective for patients with massive or torrential FTR and/or those with significant leaflet tethering [2]. For these reasons, a novel surgical or percutaneous repair option that addresses these shortcomings would be of significant value.

To test novel therapies for FTR, an *ex vivo* model of FTR is needed. Unfortunately, the currently available *ex vivo* models of the tricuspid valve are costly, difficult to replicate, or have not been formally validated [3-8]. Our laboratory has previously been successful in developing an *ex vivo* model of secondary mitral regurgitation (SMR) using isolated porcine hearts [9]. Given the comparable tricuspid anatomy between humans and swine [5-8,10] we hypothesized that porcine hearts could similarly be used to develop a static *ex vivo* model of FTR.

2. Materials and Methods

2.1. Ex vivo model setup

Isolated porcine hearts were procured from an abattoir (ATSCO, Inc, Plano, TX, USA), and any remaining pericardium was removed. The coronary arteries were ligated using a 2-0 silk suture, and the aorta and pulmonary artery were cross-clamped. Cannulae were placed into the pulmonary artery and aorta through purse string sutures and were advanced into the right ventricle and left ventricle, respectively. Pressurized air was delivered through the cannulae using a 38-W linear-drive air pump (Thomas, Gardner-Denver Medical, Sheboygan, WI, USA), and ventricular pressure was maintained at 120 mmHg in the left ventricle, and 30 mmHg in the right ventricle. Static pressurization of the left ventricle and right ventricle in such a manner results in the closure of the mitral and tricuspid valves and allows for assessment of valvular geometry (Figure 1). The right atrium was then opened and the atrial tissue was retracted laterally to allow for subsequent imaging and manipulation of the tricuspid valve (Figure 2A).

2.2. Image acquisition

A three-dimensional (3D) structured light scanner (Artec 3D, Luxembourg) was used to capture the shape and texture of the tricuspid valve surface. The scanner generates a 3D model of the tricuspid valve by projecting light onto the valvular surface and recording the pattern of light reflected back to the scanner. The light distortions caused by the surface structures of the tricuspid valve can be analyzed to generate a 3D point cloud. The point cloud is then exported into a 3D scan-to-computer-aided design reverse engineering software (Geomagic, Morrisville, North Carolina, USA), which allows for visualization of the tricuspid valve as a 3D model and enables subsequent analysis of the valvular geometry (Figure 3).

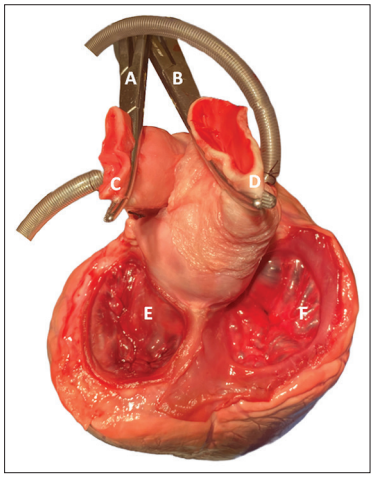

Figure 1. Pneumatic *ex vivo* model of the tricuspid valve with pulmonary artery cross-clamp (A), aortic cross-clamp (B), cannulated pulmonary artery (C), cannulated aorta (D), mitral valve (E), and tricuspid valve (F).

2.3. Induction of FTR

After imaging the tricuspid valve in its native state, the right atriotomy was closed with 4-0 prolene (Figure 2B). Closure of the atriotomy was necessary to create a closed system that could sustain right ventricular pressure even after the induction of FTR. Without this step, increases in right ventricular pressure would result in leakage of air through the tricuspid valve and loss of pressure in the right ventricle. The right ventricular pressure was then increased from 30 mmHg to

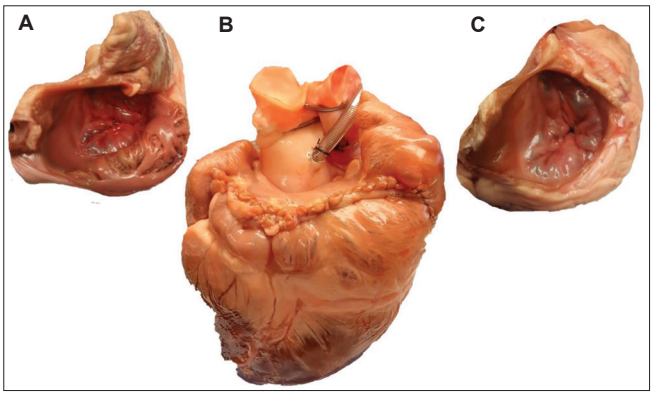

Figure 2. Development of functional tricuspid regurgitation (FTR). Representative images at each stage of development of the *ex vivo* model of FTR. (A) View of the native (control) tricuspid valve through a right atriotomy, with residual right atrial tissue retracted laterally. (B) Closure of the right atriotomy with 4-0 prolene and sustained pressurization of the right ventricle to 100 mmHg. (C) View of the regurgitant tricuspid valve with residual right atrial tissue excised.

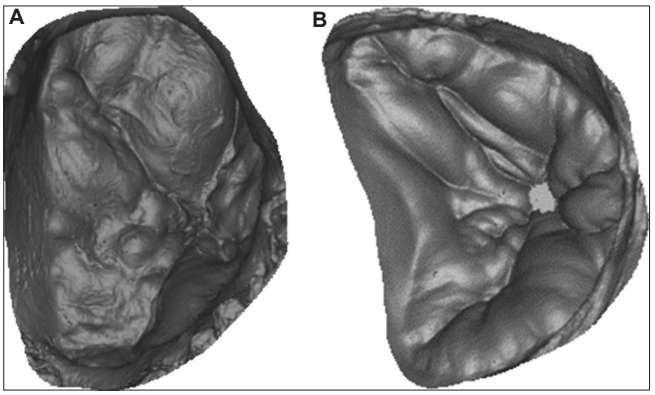

Figure 3. Representative 3D light scanner images of the tricuspid valve in the native (control) state (A), and after inducing functional tricuspid regurgitation with sustained pneumatic pressurization (B).

DOI: https://doi.org/10.36922/jctr.24.00003

100 mmHg, which mimics the right ventricular overload seen in FTR from left-sided valvular pathology. Right ventricular pressure was sustained at 100 mmHg for 3 h, with the intent of creating progressive annular and ventricular enlargement and inducing FTR. Throughout the 3-h period of sustained right ventricle pressurization, hydration of the tissues was ensured by periodically adding a small amount of fluid to the right ventricle, thus humidifying the air and maintaining the integrity of the tricuspid valve complex. Dampened towels were also applied to the exterior surface of the heart. After 3 h, the right atrium was excised to allow for optimal visualization of the tricuspid valve apparatus, and the tricuspid valve was imaged in its regurgitant state with the 3D light scanner (Figure 2C).

2.4. Outcomes

The primary outcomes of interest were tricuspid annular dimensions, including annular circumference, diameter, and area. Secondary outcomes were measures of leaflet geometry, including tenting height, angle, and area. For the model of FTR, the effective regurgitant orifice area was also measured and was defined as the area of visible malcoaptation. The annular diameter was measured in the minor axis, defined as the distance from the mid-septal leaflet to the opposite point on the annulus, and the major axis, defined as the greatest distance perpendicular to the minor axis (Figure 4A). Tenting height was defined as the maximum distance from the annular plane to the point of coaptation (Figure 4B). The tenting angle was measured as the angle between the annular plane and the septal leaflet. The tenting area was defined in the minor axis and was calculated by measuring the area between the annular plane and tricuspid leaflets. The tenting volume was defined as the volume between the annular plane and the tricuspid leaflets and was measured using a custom Python script. The Python script calculated tenting volume by dividing the valve into 100 slices along the X- and Y-axes below the annular plane, calculating the area of each slice, multiplying by the distance to the following slice, and summing these areas. The script then rotated the valve slightly for a total of 100 rotations, and the same process was repeated. After all rotations were completed, the average of all 100 tenting volumes was calculated to generate a final result.

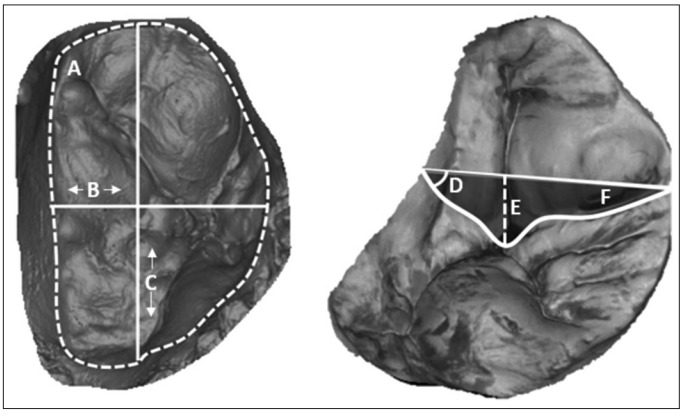

Figure 4. Geometric measurements sampled from 3D-reconstructed images of the tricuspid valve, including annular circumference and area (A), minor axis diameter (B), major axis diameter (C), tenting angle (D), tenting height (E), and tenting area (F).

2.5. Clinical validation

To compare the native geometry of the swine and human tricuspid valve and to validate the FTR model, the *ex vivo* native and FTR models were compared to publishe transesophageal echocardiographic (TEE) measurements from non-diseased (control) and FTR patients. Publications were selected if their methodology was well-described, and measurements were sampled in planes similar to those described above [11-13].

2.6. Statistical analysis

Comparisons were first made between the native and regurgitant *ex vivo* model and subsequently between the *ex vivo* model and *in vivo* echocardiographic data. Non-parametric testing was considered given the small sample size, but non-parametric comparisons between the *ex vivo* model and *in vivo* echocardiographic data were not possible as we did not have access to the underlying data sets for literature reported *in vivo* echocardiographic data. We instead verified the normality of our data using the Shapiro–Wilk test. Paired *t*-tests were then used to compare measurements between the native and regurgitant *ex vivo* model, and Student's *t*-test was used to compare measurements between the *ex vivo* model and literature-reported *in vivo* echocardiographic data. Data analysis was performed using STATA/IC 17.0 (StataCorp LLC, College Station, TX, USA), and statistical significance was set to a p-value ≤ 0.05 for all tests.

3. Results

3.1. Ex vivo model

A total of 12 porcine hearts, weighing 310–428 g each, were employed in this study. When compared to geometric measurements from the native *ex vivo* heart, all annular dimensions increased significantly with sustained pneumatic pressurization of the right ventricle (Table 1). The annular circumference and area increased by 16% and 35%, respectively (circumference: 11.8 vs. 13.7 cm, p=0.012; area: 10.5 vs. 14.0 cm^2, p=0.011). Major and minor axis diameters both increased from baseline, with the most substantial change seen in the minor axis (minor: 2.5 vs. 3.4 cm, p=0.05; major: 3.9 vs. 4.3 cm, p=0.05). The circularity index decreased with sustained pressurization (1.5 vs. 1.3, p=0.02), indicating a more circular annulus. When evaluating leaflet geometry, sustained pneumatic pressurization resulted in increased tethering of the anterior leaflet (Table 2), as evidenced by a significant increase in anterior leaflet angle (24° vs. 41°, p=0.008). Similarly, the *ex vivo* model of FTR yielded larger tenting height (8.0 vs. 11.5 mm, p=0.037), tenting area (1.0 vs. 2.0 cm^2, p=0.05), and tenting volume (3.4 vs. 7.4 cm^3, p=0.015) relative to baseline measurements. There was no significant change in septal leaflet tethering (31° vs. 30°, p=0.66). Minimal malcoaptation was present at baseline, as represented by a negligible effective regurgitant orifice area, but increased substantially after sustained pressurization (8.1 vs. 40.1 mm^2, p<0.001).

DOI: https://doi.org/10.36922/jctr.24.00003

Table 1. Comparison of clinical and *ex vivo* model annular dimensions

Annular measurements	Control	FTR	*P*-value
Annular circumference (cm)			
Ex vivo	11.8 (0.9)	13.7 (0.7)	0.01*
Clinical[a]	11 (1.5)	14 (3.8)	0.04*
P-value	0.21	0.85	
Annular area (cm^2)			
Ex vivo	10.5 (1.3)	14.1 (1.5)	0.01*
Clinical[a]	10.2 (2)	15 (5)	<0.01*
P-value	0.72	0.66	
Major axis diameter (cm)			
Ex vivo	3.9 (0.2)	4.3 (0.3)	0.05*
Clinical[a]	3.8 (0.4)	4.3 (0.8)	<0.01*
P-value	0.55	>0.9	
Minor axis diameter (cm)			
Ex vivo	2.5 (0.4)	3.4 (0.5)	0.05*
Clinical[a]	3.1 (0.5)	3.9 (0.7)	<0.01*
P-value	<0.01*	0.10	
Circularity index			
Ex vivo	1.5 (0.2)	1.3 (0.2)	0.02*
Clinical[b]	1.3 (0.1)	1.1 (0.1)	<0.01*
P-value	0.07	0.13	

Notes: Geometric measurements of the tricuspid valve annulus in an *ex vivo* porcine model in its non-diseased (control) and regurgitant (FTR) state as compared to published (clinical) TEE measurements by: [a]Karamali *et al.*[11] (n=52); or [b]Ton-Nu *et al.*[13] (n=75). All results are reported as mean (standard deviation). In the *ex vivo* model, "control" measurements were taken from the native tricuspid valve before inducing FTR. In the clinical data, "control" measurements were sampled from patients without FTR. Minor axis diameter was measured as a normal line from the mid-septal leaflet to the lateral wall. The major axis diameter was measured perpendicular to the minor axis at the point of maximum length. *$P \leq 0.05$.
Abbreviation: FTR: Functional tricuspid regurgitation.

Table 2. Comparison of clinical and *ex vivo* model leaflet geometry

Leaflet measurements	Control	FTR	*P*-value
Anterior leaflet angle			
Ex vivo	24 (7)	41 (9)	0.008*
Clinical[a]	10 (1)	25 (10)	0.003*
P-value	<0.001*	<0.001*	
Septal leaflet angle			
Ex vivo	31 (11)	30 (6)	0.66
Clinical[a]	12 (1)	22 (9)	<0.001*
P-value	<0.001*	0.040*	
Tenting height (mm)			
Ex vivo	8.0 (3.0)	11.5 (2.5)	0.037*
Clinical[a]	5.2 (1.8)	7.8 (3.4)	<0.001*
P-value	0.001*	0.013*	
Tenting area (cm^2)			
Ex vivo	1.0 (0.5)	2.0 (0.6)	0.05*
Clinical[a]	0.5 (0.3)	1.7 (1.2)	<0.001*
P-value	<0.001*	0.55	
Tenting volume (cm^3)			
Ex vivo	3.4 (1.4)	7.4 (1.4)	0.015*
Clinical[a]	1.1 (0.7)	3.0 (1.9)	<0.001*
P-value	<0.001*	<0.001*	
Effective regurgitant orifice area (mm^2)			
Ex vivo	-	40.1 (26.6)	-
Clinical[b]	-	22 (14)	-
P-value	-	0.049*	

Notes: Geometric measurements of the tricuspid valve leaflets in an *ex vivo* porcine model in its non-diseased (control) and regurgitant (FTR) state as compared to published (clinical) TEE measurements by: [a]Karamali *et al.*[11] (n=52); or [b]Florescu *et al.*[12] (n=58). All results are reported as mean (standard deviation). In the *ex vivo* model, "control" measurements were taken from the native tricuspid valve before inducing FTR. In the clinical data, "control" measurements were sampled from patients without FTR. Tenting height, area, and angle were measured in the septal-lateral plane. *$P \leq 0.05$.
Abbreviation: FTR: Functional tricuspid regurgitation.

3.2. Comparison with human echocardiographic data

When comparing the measurements obtained from the *ex vivo* porcine model to the *in vivo* human echocardiographic data, all annular measurements were comparable with the exception of baseline minor axis diameter, which was smaller in the *ex vivo* model (2.5 vs. 3.1 cm, p=0.007). Notably, after inducing FTR in the *ex vivo* model, the minor axis measurements were similar to those in humans with FTR (3.4 vs. 3.9 cm, p=0.10). When evaluating leaflet geometry, however, the majority of measurements from the *ex vivo* model differed from human echocardiographic data. At baseline, porcine leaflet geometry in the *ex vivo* model had steeper leaflet angles than those seen in normal humans (anterior: 24° vs. 10°, p<0.001, septal: 31° vs. 12°, p<0.001).

This translated into larger tenting height (8.0 vs. 5.2 mm, P = 0.001), area (1.0 vs. 0.5 cm^2, P < 0.001), and volume (3.4 vs. 1.1 cm^3, P < 0.001) relative to human measurements. These differences persisted after inducing FTR, with the exception of the tenting area, which was comparable between the *ex vivo* model and human echocardiographic data (2.0 vs. 1.7, P = 0.55). Although tenting measurements were larger in the porcine model than in human TEE data, the direction and magnitude of changes were comparable when comparing control and FTR measurements. For example, although anterior leaflet tenting angles were larger in the *ex vivo* model than in humans for both control and FTR measurements, the angle increased by an average of 17° in the *ex vivo* model and 15° in human TEE data. Similarly, tenting height in the *ex vivo* model increased by approximately 3.5 mm after sustained pressurization, as compared to a 2.6 mm increase in human TEE data.

4. Discussion

The objective of this study was to develop a simple, inexpensive, and reproducible porcine model of FTR for testing novel surgical and transcatheter interventions. We found that sustained pneumatic pressurization of the right ventricle results in geometric alterations that was comparable to those reported in the literature, including annular dilation and leaflet tethering.

After several hours of sustained right ventricular pressures at 100 mmHg, the mean tricuspid valve annular area increased from 10.5 to 14.1 cm^2 – an increase of nearly 35%. The majority of dilation occurred in the septolateral, or minor axis, corresponding to selective dilatation of the free wall of the right ventricle. These patterns are consistent with the geometric

DOI: https://doi.org/10.36922/jctr.24.00003

alterations reported in humans with FTR, wherein the normally saddle-shaped tricuspid annulus dilates and becomes more circular [14-17]. We also observed alterations in the subvalvular apparatus of the tricuspid valve, with significant increases in the anterior leaflet angle, tenting height, tenting area, and tenting volume. Historically, the importance of the subvalvular anatomy of the tricuspid valve was not appreciated, and the emphasis was placed primarily on reducing the size of the tricuspid annulus to restore leaflet coaptation. Recent publications have emphasized the importance of residual leaflet tethering as a predictor of failure after tricuspid repair and have called for novel repair strategies that address both annular dilation and leaflet tethering [18-20]. Our *ex vivo* model of FTR incorporates both of these geometric alterations and thus offers a realistic platform for testing novel repair strategies.

When directly comparing geometric measurements between the *ex vivo* model and human TTE data, the majority of annular dimensions remained similar. These results are consistent with both *ex vivo* and *in vivo* reports of porcine tricuspid anatomy. In an evaluation of 119 porcine hearts, Waziri *et al.* demonstrated no difference in tricuspid annulus circumference or area compared to humans [21]. Similarly, Fawzy *et al.* evaluated the 3D geometry of the tricuspid annulus in anesthetized swine using sonomicrometry and described similar annular dimensions to humans [22]. These findings collectively demonstrate that the native porcine tricuspid valve reasonably approximates that of a human, justifying its future utilization in translational research.

Our data demonstrated that the subvalvular apparatus of the porcine tricuspid valve was not directly comparable to that of a human. At baseline, the porcine tricuspid valve had a narrower minor axis with steeper leaflet angles and greater tenting than that of a human. As such, many of these differences persisted after inducing FTR; the porcine valve had greater anterior and septal leaflet angles, tenting height, and tenting volume relative to human TTE data. Given that our annular measurements were comparable between the *ex vivo* model and human TTE data, the observed differences in subvalvular measurements likely reflect differences in anatomy between species rather than flaws in the *ex vivo* model itself. Surprisingly, no study has evaluated the differences in subvalvular anatomy between swine and humans, despite numerous translational studies being performed in porcine models. Despite these differences, the strength of this model lies in the geometric alterations observed between the native and FTR states; although the subvalvular measurements were not identical between swine and humans, the directional and incremental changes between normal and diseased specimens were similar.

Furthermore, our model compares favorably to those described by other groups in terms of validity, simplicity, and cost. Adkins *et al.* induced FTR in ovine hearts by injecting 95% phenol around the annulus to create annular dilation, but the degree of dilation was not validated and there was no alteration of the subvalvular apparatus [3]. Stock *et al.* isolated porcine tricuspid valve complexes and mounted them on an adjustable supporting device [4]. This allowed for replication of the geometric changes observed with FTR but provided a rigid model with fixed geometry even after the application of surgical repairs. Perhaps the most comparable model was proposed by Maisano *et al.*, wherein a closed, pressurized circuit was developed using a centrifugal pump, and the porcine right ventricle was dilated with sustained pressure [23]. The authors used radiopaque markers and fluoroscopy to document the occurrence of tricuspid annular dilation, papillary muscle displacement, and induction of FTR after pressurizing the right ventricle. Our model requires fewer resources; the pneumatic pump and 3D light scanner required for this model are both commercially available and comparatively inexpensive. Furthermore, the 3D geometry of our model was directly compared to normal and diseased human TTE data in this study, whereas the model suggested by Maisano *et al.* solely describes the geometric changes relative to the baseline.

We acknowledge that this model has several limitations. First, this is a static representation of the tricuspid valve at peak systole and cannot be used to evaluate valvular anatomy in diastole. We believe that the mid-systolic phase of the cardiac cycle is the most clinically relevant to capture when evaluating the effectiveness of repair strategies for FTR. Tricuspid stenosis is exceedingly uncommon and is unlikely to occur with the application of repair strategies for FTR, where the annulus has already dilated from baseline. Even so, the diastolic performance of novel repairs would need to be examined *in vivo* or with a dynamic, pulsatile *ex vivo* model. Second, preservation of the pericardium was not possible due to the manner in which the porcine hearts were harvested. Assessment of pericardial contributions to valvular geometry would be better assessed with an *in vivo* model. Furthermore, we employed a pneumatic model for pressurizing the right ventricle, potentially resulting in different mechanics of valve closure than those observed with blood. The pneumatic model also limits our ability to assess the right ventricle, as the structured light scanner used in this model is restricted to the assessment of surface-level anatomy. Qualitative assessment of the right ventricle in the *ex vivo* model before and after sustained pneumatic pressurization suggests right ventricular dilatation as the source of increased tenting angles with the induction of FTR, but this was not quantifiable. Similarly, the individual contribution of constituent elements of the tricuspid valve was not assessed in this research. Previous research has implicated the transition between the papillary muscle and chordae tendinae as a potential origin of valve deformation [24,25], which may or may not be accurately represented by the *ex vivo* model proposed herein. Finally, as mentioned above, it is important to take into consideration the baseline differences in anatomy between swine and humans when interpreting results from this model. We observed modest differences in subvalvular anatomy between swine and human data. As such, any subvalvular geometric measurements sampled in the *ex vivo* FTR model should be interpreted in reference to *ex vivo* native measurements, and not to normal human TTE data.

5. Conclusion

The *ex vivo* porcine model of FTR characterized in this study demonstrates consistent annular dilation and leaflet

DOI: https://doi.org/10.36922/jctr.24.00003

tethering that mimics the direction and magnitude of geometric alterations observed in humans with FTR. This model offers a simple, reproducible, and cost-effective option for testing and optimizing novel interventions for FTR before *in vivo* or clinical studies.

Acknowledgments

None.

Funding

This research was funded, in part, by the American Heart Association (Award #908805) and the American Association for Thoracic Surgery Summer Intern Scholarship.

Conflict of Interest

The authors have no competing interests to disclose.

Ethics Approval and Consent to Participate

This study did not involve human subjects and was therefore exempt from IRB approval.

Consent for Publication

Not applicable.

Availability of Data

Data are available from the corresponding author upon reasonable request.

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DOI: https://doi.org/10.36922/jctr.24.00003

Journal of Clinical and Translational Research 2024; 10(3): 219-228

Journal of Clinical and Translational Research

Journal homepage: http://www.jctres.com/en/home

ORIGINAL ARTICLE

The impact of mode of birth delivery on child health in India

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ARTICLE INFO

Article history:
Received: December 27, 2022
Accepted: March 4, 2024
Published Online: June 5, 2024

Keywords:
Child growth
Life table
Multivariate regression
Stunting
Neonatal mortality
Infant mortality

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ABSTRACT

Background: A skilled birth attendant and the place of delivery have significant effects on child growth.
Aims: The present paper aims to examine the mode of delivery and its impact on child health among children (0 – 59 months) in India.
Methods: The life table estimation of mortality and both bivariate and multivariate logistic regressions were used to identify the association between child health and mode of delivery using data from the National Family Health Survey conducted in 2015 – 2016.
Results: After adjusting for socioeconomic and biodemographic factors, poor child growth (measured through Z-scores for stunting, wasting, and underweight categories) was more significant in cesarean delivery compared to normal delivery. In contrast, live birth for different groups of women was reportedly higher in normal vaginal delivery than in cesarean delivery. Neonatal and infant mortality rates were lower for normal delivery than cesarean delivery, particularly in public hospitals. The risk of child death was also higher in cesarean delivery, particularly in the neonatal period.
Conclusion: The findings from this study could inform the development of health-care policies and the implementation of strategies aimed at improving the quality of painless labor and prompt delivery in health-care facilities, particularly public hospitals.
Relevance for Patients: The present study may help pregnant women and their providers decide whether a cesarean delivery is appropriate.

1. Introduction

Child malnutrition and mortality represent major public health challenges, particularly in low- and middle-developing countries like India. In 2017, nearly 151 million (22%) of children reported stunted growth [1], and 45% of global child deaths were among children <5 years old [2,3]. The major causes of child malnutrition and mortality in low and middle-developing countries are poor nutrition, infectious diseases, household environment, and different modes of birth delivery [4]. In general, human birth can occur through natural delivery, assisted delivery, or cesarean section, with the latter sometimes performed due to social factors [5]. Several studies have suggested that cesarean sections can have a negative impact on both maternal and child health outcomes [6-11]. The World Health Organization (WHO) recommended that the utilization of cesarean section should be limited to 5 – 15% in any population to avoid any negative health impact [12-17]. A cesarean section rate below 5% implies that a substantial proportion of women experience successful delivery without surgical complications, indicating adequate access to skilled delivery services [18]. In addition, it is indicative of favorably saving both infant and maternal lives during emergency obstetric situations and has also contributed to reductions in maternal and neonatal mortality, as well as morbidity [19].

DOI: https://doi.org/10.36922/jctr.22.00239

Conversely, cesarean rates above 15% suggest an increased risk of mortality, financial burden, and clinical risks on the health of both mother and baby, as well as the health-care system [20-22]. The previous studies conducted in high-income countries have examined how women with various obstetric histories may influence the likelihood of cesarean delivery and its impact on neonatal and infant mortality rates [23,24]. Polidano *et al.* suggested that cesarean birth is associated with negative cognitive growth of the child [25], such as the development of asthma, Type I diabetes, allergies [26-28], and obesity [29], and is also correlated with poor academic performance [30,31]. Infants born vaginally pass through the birth canal directly, whereas cesarean-born infants come into contact with the mother's skin through assistance from doctors or nurses in the hospital. An early study by Rowe-Murray and Fisher found that cesarean-born infants are less likely to immediately come into contact with the mother's skin after birth, and they also reported delayed breastfeeding, that is, after 24 h in post-delivery [32].

Globally, cesarean birth rates have increased and vary across different countries due to diverse socioeconomic factors and differential health-care services. Moreover, cesarean birth rates are higher in Asian countries compared to other countries. In Ghana, the cesarean birth rate increased from 3% to 23% between 2003 and 2014 [33,34]; in Iran, cesarean section operations contribute to 40.0% of all births [35]; in China, the cesarean birth rate has reached 34.9% [36]; and in Brazil, the cesarean birth rate accounts for 56%, corresponding to approximately 90% in the private sector [37]. In India, the Fourth National Family Health Survey (NFHS-4) reported an increase in cesarean delivery rates from 8.5% to 17.2% between 2005 and 2016, but this was still lower compared to other developing countries such as Brazil and China [38]. As reported in an earlier study, women from higher-educated and wealthier backgrounds are more likely to undergo cesarean sections than women from less-educated and lower-income families. Furthermore, women admitted to private health institutions are more likely to have cesarean births than those admitted to government-owned health facilities [39].

There are currently more debates on the surgical procedures involved in birth delivery among women in private and public health institutions. Some studies suggested normal vaginal delivery [40,41], whereas others recommended cesarean delivery [42]. Vaginal delivery is reportedly more commonly associated with postpartum hemorrhage [41], whereas postpartum morbidity occurs more often in cesarean delivery [40]. In comparison with cesarean delivery, normal vaginal delivery is a physiological process of human reproduction and has many positive effects. For example, first contact with the mother and early breastfeeding is important for the child's psychological development [43]. Conversely, cesarean birth is an unnatural mode of delivery and is associated with an increased risk of endometritis, pneumonia, and other conditions, leading to poorer psychological development of newborns [5,41]. Therefore, the present paper aims to identify the impact of different birth delivery methods on child growth.

2. Methods

2.1. Data collection

The present study utilized data from NFHS-4, which was conducted in 2015 – 2016 by the Ministry of Health and Family Welfare. The NFHS is one of the important large-scale biodemographic and health surveys in India, providing sufficient information on fertility, mortality, nutritional status, family planning, and health-care utilization. The sample size of the survey included 259,627 birth records from the 5 years preceding the survey. During the survey, all women (aged 15 – 49 years) provided comprehensive birth histories, including the sex, delivery date, and survival status of each newborn. Detailed information on the survey is available in the national report [38].

2.2. Outcome variables

Neonatal and infant mortalities were two dependent variables evaluated in the study. Neonatal mortality is defined as the death of newborn babies within 28 days of birth. Infant mortality is defined as the death of newborn babies before reaching 12 months. Another dependent variable evaluated in the study was child growth, which was categorized into stunting, wasting, and underweight. Stunting refers to children (aged 0 – 59 months) whose height-for-age Z-score is <−2 standard deviation (−2*SD*) (i.e., $Z < -2SD$) below the median of the reference population. Likewise, underweight and wasting refer to children (aged 0 – 59 months) whose weight for age and height Z-scores are <−2*SD* (i.e., $Z < -2SD$) below the median of the reference population. These indicative Z-scores were then computed based on the WHO-recommended reference population [44]. The above variables were classified as absent if $Z \geq -2SD$. The mortality variables were coded as 1 if the baby had died and 0 if the baby survived.

2.3. Explanatory variables

The place of delivery was considered a primary independent variable in the study. According to NFHS-4, the place of delivery could be categorized into: (i) Public hospital (government hospital, government health center, government health post, or other public sector) and (ii) private medical sector (private hospital or clinic and other private medical facility). We also categorized the delivery method accordingly: Cesarean section or normal vaginal delivery. Based on the reviewed literature, we investigated several biodemographic and socioeconomic variables that could also significantly impact a child's health [45-48], including the mother's age during childbirth (15 – 19, 20 – 29, 30 – 39 years, or 40 – 49 years), preceding birth interval (<24 or ≥24 months), birth order (1, 2, or 3), place of residence (urban or rural), household wealth (poorest, poorer, middle, richer, or richest), and birth attendant (doctors or nurses).

2.4. Statistical analysis

A comparative analysis was performed to evaluate the effect of different delivery methods (normal vs. cesarean) on child

DOI: https://doi.org/10.36922/jctr.22.00239

growth. The life-table technique was developed to estimate neonatal and infant mortality rates based on birth history variables collected from the Child Mortality Census dataset. Binary logistic regression models were used to identify the odds of normal and cesarean delivery. Childbirth through cesarean section and normal delivery were coded as 1 and 0, respectively. Following the collection of bivariate data, multivariate logistic regression models were constructed for each of the dependent variables. The results of the regression analysis were presented as odds ratios (OR), along with the corresponding 95% confidence intervals (CIs). All statistical analyses were performed using STATA® software (version 15.0).

3. Results

Table 1 presents the total number of births delivered through different delivery methods and places of delivery, along with their background characteristics. Mothers in the 15 – 19 years age group reported the highest number of births through normal delivery in both public (i.e., 98% normal birth vs. 3% cesarean birth) and private hospitals (i.e., 67% normal birth vs. 33% cesarean birth). Notably, older mothers (e.g., >30 years) reported higher cesarean births than normal births in both private and public hospitals, most likely due to their capability and means compared to young mothers (i.e., <19 years). Mothers who gave birth more than 24 months after a preceding birth were more inclined toward cesarean section than those who had given birth less than 24 months after a preceding birth. Interestingly, birth order was negatively correlated with cesarean section and positively correlated with normal delivery. This could be associated with Muslim families who prefer normal delivery over cesarean section. Cesarean sections were reportedly more common in urban residences compared to rural residences (42.0% vs. 35.5%) due to the availability and accessibility of medical facilities and transportation. Household wealth plays a dominant role in determining the birth delivery method. The "richest" households would prefer cesarean birth more than the "poorest" households (43.8% vs. 24.3%, respectively). The differences between normal and cesarean births in the "richest" households in private and public hospitals were 12% and 57%, while the differences in the "poorest" households were much wider at 52% vs. 92%, respectively. In both private and public hospitals, most cesarean births were delivered by doctors, while most normal births were delivered by the nurse.

Table 2 presents the neonatal and infant mortality rates by different birth delivery methods in public and private hospitals along with their biodemographic and socioeconomic characteristics. Results indicate that neonatal and infant mortalities varied across the socioeconomic and biodemographic

Table 1. Total number of births (%) based on the delivery method and place of delivery according to different biodemographic and socioeconomic backgrounds in India (2015 – 2016)

Background	Number of births, *n* (%)			
	Private hospital		Public hospital	
	Cesarean	Normal	Cesarean	Normal
Mother's age (years)				
15 – 19	399 (33.3)	798 (66.7)	391 (3.4)	3764 (97.6)
20 – 24	5525 (34.6)	10424 (65.4)	4564 (9.9)	41497 (90.1)
25 – 29	8179 (38.0)	13338 (62.0)	5891 (10.8)	48428 (89.2)
>30	6585 (42.0)	9090 (58.0)	4782 (13.1)	31711 (86.9)
Preceding birth interval (months)				
<24	1950 (29.3)	4712 (70.7)	1674 (7.5)	20553 (92.5)
≥24	18738 (39.3)	28938 (60.7)	13954 (11.7)	104847 (88.3)
Birth order				
1	11066 (42.1)	15197 (57.9)	8067 (14.0)	47923 (86.0)
2	6899 (39.5)	10559 (60.5)	5399 (12.1)	39201 (87.9)
>3	2723 (25.7)	7894 (74.3)	2162 (5.3)	38276 (94.7)
Place of residences				
Rural	11307 (35.5)	20712 (64.5)	10197 (9.2)	100062 (90.8)
Urban	9381 (42.0)	12938 (58.0)	5431 (17.6)	25338 (82.4)
Household wealth				
Poorest	1102 (24.3)	3249 (75.7)	1417 (4.0)	33661 (96.0)
Poorer	2034 (28.9)	4983 (71.1)	2822 (7.8)	33546 (92.2)
Middle	3745 (36.0)	6648 (64.0)	4208 (13.3)	27386 (86.7)
Richer	5703 (40.5)	8377 (59.5)	4178 (17.6)	19597 (82.4)
Richest	8104 (43.8)	10393 (56.2)	3003 (21.1)	11210 (78.9)
Delivered by				
Doctor	18509 (42.3)	25237 (57.7)	13184 (16.3)	67825 (88.9)
Nurse	11613 (35.9)	20662 (64.1)	9126 (9.0)	92869 (91.0)

DOI: https://doi.org/10.36922/jctr.22.00239

Table 2. Neonatal and infant mortality rates (*n* per 1000 live births) based on the delivery method and place of delivery according to different demographic and socioeconomic backgrounds in India (2015 – 2016)

Background	Mortality, *n* per 1000 live births							
	Neonatal				Infant			
	Private hospital		Public hospital		Private hospital		Public hospital	
	Cesarean	Normal	Cesarean	Normal	Cesarean	Normal	Cesarean	Normal
Mother's age (years)								
15 – 19	28	61	41	34	36	72	51	55
20 – 24	21	35	31	28	27	44	44	40
25 – 29	20	24	30	24	26	31	38	34
>30	21	27	31	25	27	35	42	37
Preceding birth interval (months)								
<24	28	48	27	34	37	62	37	53
≥24	20	26	32	24	26	33	42	34
Birth order								
1	19	29	37	30	24	36	46	40
2	17	22	22	21	24	28	32	31
>3	43	39	32	27	53	52	50	41
Place of residence								
Rural	28	37	36	28	35	46	48	40
Urban	13	18	24	19	18	23	31	29
Household wealth								
Poorest	56	59	60	35	64	73	84	49
Poorer	41	49	38	28	53	63	50	40
Middle	25	31	30	22	33	41	41	34
Richer	18	23	22	20	24	27	30	27
Richest	11	15	25	13	15	20	30	19
Delivered by								
Doctor	20	25	27	25	26	32	38	34
Nurse	21	33	32	25	28	42	42	36

characteristics for different birth delivery methods and places of delivery. Both neonatal and infant mortality rates were reportedly lower for cesarean births than normal births in both private and public hospitals. Mothers who gave birth at ages 15 – 19 years old reported higher infant and neonatal mortality rates compared to those who gave birth at ages 25 – 29 years old. Similarly, the mortality rate was also high for mother's aged above 30 years old for both normal and cesarean births in public and private hospitals. Higher birth order is associated with higher rates of infant and neonatal mortalities for cesarean births compared to normal births in both public and private hospitals. The infant mortality rate in rural residences was higher compared to urban residences. Likewise, the infant mortality rate was higher in private hospitals compared to public hospitals for normal births in rural residences. The "richest" households had lower rates of neonatal and infant mortalities compared to the "poorest" households. Similarly, the mortality rate is also lower for births delivered by doctors compared to nurses.

Table 3 displays overall child growth according to different birth delivery methods. It was found that child malnutrition (i.e., stunting, wasting, and underweight) was lower for normal births compared to cesarean births. For example, stunted child growth from cesarean and normal births was 40% and 27%, respectively (Figure 1). Similarly, wasted children from cesarean and normal births were 21% and 17%, while underweight children from cesarean and normal births were 36% and 23%, respectively.

Table 3. Child growth indicators according to the birth delivery method

Child growth	Delivery method	%	*SD*	95% CI	χ^2	*P*
Stunting	Cesarean	40.2	0.5	(0.40 – 0.40)	2.0	0.000
	Normal	27.1	0.4	(0.26 – 0.27)		
Wasting	Cesarean	21.0	0.4	(0.20 – 0.21)	310.0	0.000
	Normal	16.8	0.4	(0.16 – 0.17)		
Underweight	Cesarean	36.3	0.5	(0.36 – 0.36)	2.1	0.000
	Normal	23.2	0.42	(0.22 – 0.23)		

Abbreviations: CI: Confidence interval; *SD*: Standard deviation.

Table 4 presents the ORs for neonatal and infant mortalities of cesarean and normal births along with their background characteristics. Results suggested that the ORs of neonatal and infant mortalities of cesarean births were 0.24 and 0.28 times, respectively, lower than normal births in public and private hospitals. The differences in risk of neonatal and infant mortalities for cesarean births were negligible between

DOI: https://doi.org/10.36922/jctr.22.00239

private and public hospitals. Conversely, neonatal mortalities were 0.88 times lower in public hospitals compared to private hospitals for normal births. This could be due to the preferences of private hospital doctors to perform cesarean sections over normal deliveries, thereby significantly increasing neonatal and infant mortalities of normal births in private hospitals. With mothers aged 15 – 19 years old during delivery as the reference category, it was observed that mothers aged 25 – 29 years old reported a lower risk of neonatal mortality from normal births compared to cesarean births. Mothers, who gave birth more than 24 months after a preceding birth, reported significantly lower risks of neonatal and infant mortalities compared to those who gave birth less than 24 months after a preceding birth. Birth orders of more than the third births were at significantly higher risks of neonatal and infant mortalities (i.e., 1.23 and 1.32 times, respectively) for cesarean births compared to normal births. Newborns from the "poorer" households were at a higher risk of neonatal and infant mortalities compared to newborns from the "richest" households for both normal and cesarean births. In addition, the risk of neonatal mortality was significantly lower for normal births delivered by doctors than nurses (OR: 0.91 vs. 0.93).

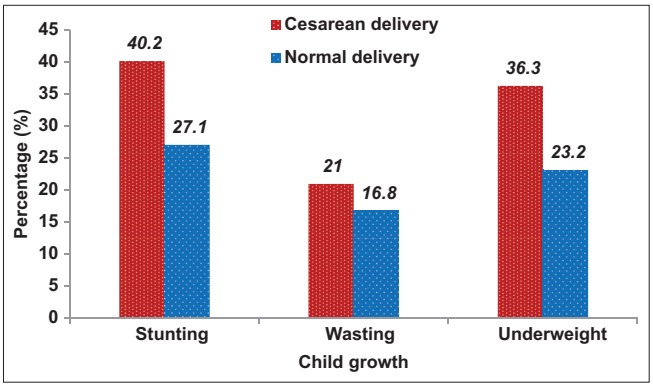

Figure 1. Impact of birth delivery method on child growth.

4. Discussion

The effect of cesarean deliveries on child health and the higher cost associated with cesarean deliveries compared to

Table 4. OR and 95% CI for neonatal and infant mortalities based on the delivery method according to different demographic and socioeconomic backgrounds in India (2015 – 2016)

Background	Mortality, OR (95% CI)			
	Neonatal		Infant	
	Cesarean	Normal	Cesarean	Normal
Place of delivery				
Public	0.24 (0.17 – 0.34)***	0.88 (0.81 – 0.96)***	0.28 (0.20 – 0.39)***	0.89 (0.83 – 0.95)***
Private	0.25 (0.17 – 0.35)***	1.33 (1.21 – 1.47)***	0.28 (0.20 – 0.39)***	1.20 (1.10 – 1.31)***
Mother's age (years)				
15 – 19®	-	-	-	-
20 – 24	0.83 (0.57 – 1.19)	0.77 (0.68 – 0.88)***	0.89 (0.63 – 1.25)	0.73 (0.65 – 0.82)***
25 – 29	0.78 (0.54 – 1.13)	0.64 (0.56 – 0.73)***	0.86 (0.61 – 1.21)	0.62 (0.56 – 0.70)***
>30	0.77 (0.52 – 1.13)	0.68 (0.60 – 0.79)***	0.86 (0.60 – 1.23)	0.68 (0.61 – 0.77)***
Preceding birth interval (months)				
<24®	-	-	-	-
≥24	0.74 (0.61 – 0.91)***	0.53 (0.50 – 0.57)***	0.77 (0.64 – 0.92) ***	0.53 (0.50 – 0.56)***
Birth order				
1®	-	-	-	-
2	0.74 (0.63 – 0.88)***	0.58 (0.54 – 0.62)***	0.84 (0.72 – 0.96) **	0.61 (0.58 – 0.65)***
>3	1.23 (1.01 – 1.49)**	0.70 (0.65 – 0.76)***	1.32 (1.11 – 1.56) ***	0.80 (0.75 – 0.85)***
Place of residence				
Urban®	-	-	-	-
Rural	1.05 (0.90 – 1.22)	1.02 (0.95 – 1.10)	1.04 (0.91 – 1.18)	1.01 (0.94 – 1.07)
Household wealth				
Poorest®	-	-	-	-
Poorer	0.82 (0.66 – 1.02)*	0.84 (0.79 – 0.89)***	0.82 (0.67 – 1.00)*	0.86 (0.82 – 0.91)***
Middle	0.60 (0.48 – 0.74)***	0.68 (0.64 – 0.74)***	0.61 (0.50 – 0.74)***	0.73 (0.69 – 0.78)***
Richer	0.45 (0.36 – 0.57)***	0.57 (0.52 – 0.63)***	0.47 (0.38 – 0.57)***	0.62 (0.57 – 0.67)***
Richest	0.37 (0.29 – 0.47)***	0.40 (0.36 – 0.46)***	0.36 (0.29 – 0.44)***	0.44 (0.39 – 0.48)***
Birth delivered by				
Doctor®	0.79 (0.66 – 0.94)**	0.91 (0.88 – 1.00)**	0.81 (0.70 – 0.95)***	0.89 (0.84 – 0.93)***
Nurse	0.99 (0.86 – 1.13)	0.93 (0.87 – 1.00)**	0.95 (0.85 – 1.07)	0.94 (0.89 – 0.99)**

®Denotes the reference category; ***P<0.01; **P<0.05; *P<0.10.
Abbreviations: CI: Confidence interval; *SD*: Standard deviation.

DOI: https://doi.org/10.36922/jctr.22.00239

normal deliveries in the private sector are significant issues and challenges today. The present study investigated the different birth delivery methods and their risks to child growth and mortality in India using the current nationally representative data from NFHS-4 (2015 – 2016). Our findings revealed disparities in different birth delivery methods across the various socioeconomic and biodemographic characteristics in India. Women aged 25 – 29 years old were more likely to undergo cesarean delivery compared to those aged 15 – 19 years old. Similarly, pregnant women of more than 24 months of a preceding birth were more likely to opt for cesarean section compared to those of <24 months of a preceding birth. Newborns of higher birth orders (>3) were less likely to be delivered through cesarean section than normal vaginal delivery. In addition, women who only want a single child are more likely to opt for a cesarean section for their pregnancy than those who are expecting two or more childbirths. This was emphasized in earlier studies based on the women's perceptions regarding the efficacy of the cesarean procedure as a means to ensure newborn survival and to avert the risks of birth complications or stillbirth [49]. Women from urban residences were more likely to opt for cesarean section for childbirth compared to rural residences. This preference among urban women could be associated with the easy accessibility and availability of healthcare (public and private hospitals) facilities for maternal and child health services [49]. Women from the "richest" household's preferred cesarean delivery compared to those from the "poorest" households. Household wealth and education are attributed to the female autonomy to develop greater confidence and decision-making power regarding their health [50,51]. An earlier study also suggested that educated women from the "richest" households have access to higher quality services and health-care facilities compared to other less-privileged women [52]. Our study also demonstrated that most childbirth in public hospitals was delivered by normal vaginal delivery than cesarean delivery. In general, in public hospitals, doctors prefer cesarean delivery, especially for complicated pregnancies, including abnormal labor pain and postpartum hemorrhage. In many high- and middle-income countries, cesarean births are more common than normal births in private institutions [53,54]. An earlier study investigating the short- and long-term effects of cesarean section on women and child health suggested that normal vaginal delivery reduced the length of hospital stay, financial cost, and the risk of hysterectomy for postpartum hemorrhage, while cesarean delivery reduced the risk of vaginal injury, abdominal and perineal pain during birth and 3 days postpartum, early postpartum hemorrhage, and obstetric shock [55].

Our study also analyzed the effect of different birth delivery methods on neonatal and infant mortalities and revealed that neonatal and infant mortality rates varied across socioeconomic characteristics. Neonatal and infant mortality rates were lower for cesarean births in private institutions compared to public institutions. Moreover, cesarean delivery plays a dominant role in the survival of newborns and prevents perinatal mortality and severe morbidity, such as intrapartum asphyxia [56]. Another systematic review validated the association between cesarean birth and mortality and concluded that cesarean birth improves maternal, neonatal, and infant survival by 9 – 16%, but the different socioeconomic factors could be a varied association between cesarean birth and mortality [57]. Betran *et al.* indicated that the risk of newborn mortality is higher when vaginal delivery is performed by untrained medical staff (inexperienced or having inadequate skill), while planned cesarean delivery is the safest option for mothers and babies [56]. Our findings revealed that Indian women do not have adequate access to cesarean services, most likely due to insufficient provision of equipment, lack of emergency room for obstetrics services, lack of skilled birth attendants, untrained medical staff, major geographical barriers (e.g., long-distance), and lack of transportation [58,59].

Analysis of child growth (i.e., stunting, wasting, and underweight) based on different birth delivery methods displayed a negative association between cesarean births and healthy child growth, such as the influence on feeding practices [60]. The study also revealed a higher prevalence of stunted, wasted, and underweight child growth from cesarean births compared to normal vaginal births. A prior study also suggested that cesarean births may have negative implications related to neuropsychiatric disorders and mother-infant relationships [5]. Another study implied that schizophrenia and mental disorders were 10 times higher among children born through cesarean section [61]. A growing number of studies reported that these children born through cesarean section had poorer sensory integration ability than those born by natural vaginal birth [62-65]. A study by Evans *et al.* reported a significantly faster transfer of breast milk from mother to child in vaginal birth than in cesarean births in the first 5 days postpartum [66]. Similarly, Scott *et al.* also found that delayed onset of lactation was significantly higher for cesarean births compared to normal vaginal births [67].

The logistic regression model examined significant predictors of neonatal and infant mortalities based on different birth delivery methods. After adjusting for potential confounding factors, we identified that the mother's age during delivery, preceding birth interval, birth order, place of residence, wealth index, and skilled birth attendants were significantly associated with the decisions on selecting cesarean or normal vaginal delivery methods. The findings suggest that older mothers during delivery and longer preceding birth intervals reduced the risk of neonatal and infant mortalities from cesarean births. Furthermore, newborns of higher birth orders had higher risks of dying from cesarean delivery compared to normal vaginal delivery. Consistent with previous studies, our findings indicated that women from the "richest" wealth quintile had lower risks of infant mortality than those from the "poorest" households [68,69]. The study confirmed that poverty is the major factor responsible for the reduced odds of newborn mortality from cesarean births. In addition, the higher rate of cesarean deliveries is often attributed to higher costs of healthcare, which also impacts the economic burden of a household as measured by the wealth index.

The major strength of this study is the utilization of nationally representative data, which corresponds to a large sample size that evaluates normal and cesarean births in public and private institutions. This study had several limitations: (i) the data lacked information relating to the clinical indications of cesarean

DOI: https://doi.org/10.36922/jctr.22.00239

section, such as the lack of distinguishment between elective and emergency cesarean sections; (ii) various socioeconomic and biodemographic factors were included in the study, but women decision making power was not considered in the study, which would significantly influence in the decision on delivery practice; (iii) the study did not cover the accessibility (e.g., the number of primary healthcare centers, subcenters, and community centers) and quality (e.g., number of doctors and beds) of healthcare facilities, which might influence the decision on healthcare delivery; and (iv) there are insufficient data on the physical barriers, such as distance from health centers, transportation, and road facilities. Notwithstanding, the study has provided important insights into the association between child growth and different birth delivery methods.

5. Conclusion

Cesarean births may have adverse effects on child growth and increase the risk of mortality compared to normal vaginal births. The differences in the prevalence of cesarean births between public and private institutions may be due to the difference in prenatal and delivery care between these two settings, and this could influence the delivery outcome or the preference for a delivery method [70]. The low rate of cesarean delivery in the private sector is highly associated with several socioeconomic factors. Therefore, health policies and programs should aim to improve reproductive and child health-care services, with a particular focus on enhancing the quality of obstetric care, especially for cesarean sections [71]. Furthermore, efforts should focus on improving the quality of painless labor and vaginal delivery in both public and private health-care institutions to reduce the number of cesarean births.

Acknowledgments

None.

Funding

None.

Conflict of Interest

The authors declare no conflicts of interest in this research.

Ethics Approval and Consent to Participate

Not applicable.

Consent for Publication

Not applicable.

Availability of Data

Data are available from the corresponding author on reasonable request.

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DOI: https://doi.org/10.36922/jctr.22.00239

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