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Full Length Research

Empirical Analysis of Enteric Pathogens in Raw Milk Sold at Aduwawa, Agbor,Asaba, Auchi and Warri, Nigeria.

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Abstract: This study aims to investigate the prevalence of enteric pathogens in raw milk sold at Warri, Agbor, Asaba, Aduwawa and Auchi. A total of 30(fifteen samples collected twice) raw milk samples were collected from market vendors and cattle ranch in the above locations and transported in universal bottles in an ice cooler within twenty four hours to the laboratory for analysis. Bacteria identification, colony morphology & biochemical test was carried out to identify the microorganism, antibiotic sensitivity was also carried out using nutrient agar and multi-drug-disc. In the thirty samples analyzed, Escherichia coli was prevalent in all locations (31.3%, 28.5%, 42.9%, 35.7%, 35.7%) respectively for the first batch of samples analyzed. The second batch of samples analyzed indicated the following results (27.7%, 35.7%, 29.4%, 31.2%, 29.4%) respectively. The occurrence of these pathogens *(Pseudomonas sp., E.coli, Proteus, Klebsiella, Salmonella and Enterobacter*) in raw milk are due to several factors such as animal health, poor milking practices, and poor animal waste management. Taken together, qualitative and quantitative findings revealed that in order to ensure safety of raw milk, regulatory authorities should establish guidelines and standards based on research findings to cover the entire raw milk chain across Delta and Edo state to alleviate the risk associated with disease outbreak of raw milk.
Keywords: Raw milk: Colony: Samples: Cattle: Gastroenteritis: Health: Nutrition: Nigeria.

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1.0 Introduction of the Study

Raw milk is milk that has not been heat treated. Milk is a complex biological fluid & by its nature, a good growth medium for many micro- organisms. Because of its specific production it is impossible to avoid contamination of milk with micro-organisms therefore the microbial content of milk is a major feature in determining its quality (Rogelj, 2003; Okorie, 2014). Bacterial contamination of raw milk can originate from different sources: air, milking equipment, feed, soil, feces and grass (Coorevits et al., 2008). The number and types of micro-organisms in milk immediately after milking are affected by factors such as animal & equipment cleanliness, season, feed & animal health (Rogelj, 2003). It is hypothesized that differences in feeding and housing strategies of cows may influence the microbial quality of milk (Coorevits et al., 2008). Rinsing water for milking machine and milking equipment washing also involve some of the reasons for the presence of a higher number of micro-organisms including pathogens in raw milk (Bramley, 1990). After milking, milk is cooled, which additionally influence the dynamic of microbial process (Rogelj, 2003).

Milk & milk-products provide a wealth of nutritional benefits however; raw milk can harbor dangerous microorganisms which may pose serious health risks to humans. Over 200 known diseases are transmitted through eating food contaminated by a variety of agents including bacteria, parasite, viruses, and fungi (Oliver et al., 2005) Milking and milk handling practices in informal sector are done commonly without observing hygienic practices. It is a common practice to vend milk in inappropriate milk holding & storage equipment. Such practice poses a threat to public health as chances of consuming unsafe milk are very high. Since there is little or no quality control for milk produced and handled in the informal channels, there is potential risk of contamination by zoonotic pathogens, adulterants, enteric pathogen & antimicrobial drug residues hence public health risks to consumers (Kurwijila et al., 2006), since it's a common practice to drink raw unpasteurized milk in some parts of Nigeria. Some people prefer drinking raw milk believing that they have advantages and value such as taste and convenience over the pasteurized one (Altalhi & Hassan, 2009; Angulo et al., 2009).

Enteric pathogens involved in causing food borne diseases due to the consumption of raw milk include *Escherichia coli, Listeriamonocytogenes, Salmonella, Campylobacter, Proteus, Klebsiella* and *Clostridium botulinum*. If these pathogenic bacteria are present in raw milk, it is of major public health concern, especially for those individuals who drink raw milk frequently (Chye et al., 2004). Salmonella food poisoning is one of the most common and widely distributed diseases in the world, estimated to cause 1.3 billion cases of gastroenteritis and three million deaths worldwide (Ohud et al., 2012). E. coli is frequently a contaminating organism compared to other microbes and it is a reliable indicator of fecal contamination (Kumar and Prasad, 2010). The aim and objective of this study is therefore to determine the microbial population of enteric pathogens in raw milk and to investigate the prevalence of enteric pathogens in raw milk sold at Auchi, Aduwawa, Asaba, Agbor and Warri.

2.0 Background of the Study

Contaminated milk is a health risk & reduces the chances of good quality production of milk and milk- based products. Dairy production systems are divided into two main types n namely the extensive and intensive system.

2.1 Extensive or Traditional System

Under the extensive system, the producers are generally scattered among rural communities at some considerable distance from the urban centers (Okorie, 2014). The stock used consists of a collection of cows sometimes goats and sheep. The cows are not selected for high milk production or any of the other characters derivable in a good dairy animal (Ubreye Benjamin et al., 2022; Ukonu et al., 2022; Owolabi et al., 2022). Milking is not carried out at regular intervals and very often there is no record for milk produced by each cow. There are no cultivated pastures on which to feed the animals. The animals rely on grazing on the open range grounds with the change of seasons. In most cases, this development results in a very low level of production.

2.2 Intensive (modern) System

This system involves the use of dairy animals specialized for milk production. This involves the investment of considerable capital. The size of the dairy herd could vary from 50-100 cows for small scale operations and up to 500-1000 cows for medium size operation (Eshiett et al., 2022; Osho & Haruna, 2022). The large scale operation has more than 1000 cows. The animal used for this operation are high yielding European type of breeds e.g. Friesian. In some urban dairies in Nigeria, crosses of European breeds with indigenous cattle, selected indigenous cows are used in urban dairies in Nigeria. Breeding records are kept and selection for high milk yield is intensively carried out. The milk is regularly tested for quality. The animals are fed regularly on cultivated pastures

usually green soiled or zero-grazed. The animals are housed and milking is usually done in a dairy Parlor under hygienic conditions. There is a considerable degree of mechanization in most of the operations. The animals are subjected to regular veterinary inspection to prevent and cure diseases.

In most temperate countries, milk comes mainly from domesticated cattle which have over several generations become specialized for milk production e.g *Holstein-Friesian*, *Jersey and* Indigenous dairy breeds such as the kuri, shuwa arab & white Fulani. Most of the indigenous breeds particularly, the Southern breeds offered low prospect for milk production due to poor performances in experimental stations (Okorie, 2014).Most of the human enteric pathogens being assessed can originate from clinically healthy animals from which milk is obtained. Pathogenic bacteria can enter milk from several animal sources including direct passage from blood to the milk, mastitis, and fecal contamination during or after milking; from human skin; and the environment (LeJeune & Rajala-Schultz, 2009). Dairy farms on their own are an important reservoir of food borne pathogens (Olver*et al.*, 2005). The relative importance of the various sources of contamination depends on the farming practices and may be different for each of the pathogens. The relationship between bacteria and ill-health in adult dairy cattle is highly variable. *Salmonella* spp. is a major cause of ill-health in adult dairy cattle (Low *et al.*, 1997), and may be shed in the feces of both diseased and unaffected individuals. It follows that the prevention and control of these agents through appropriate herd health management schemes will not only reduce the incidence of clinical disease, but also reduce herd prevalence and fecal shedding in clinically normal animals that may be a source of contamination of raw milk (Ruegg, 2003).Poor, unhygienic milking practices, soiled udders and teats, damaged teats, & poor operator hygiene can all lead to increased contamination of raw milk (Blowey & Edmondson, 2010). A study of 235 dairy herds on Prince Edward Island (PEI) identified pre-milking udder preparation as an important determinant of a range of different bacterial counts in milk (Elmoslemany *et al.*, 2010). The amount of soiling on the teats prior to milking and the method of udder preparation prior to milking were associated with total aerobic count (TAC). This is consistent with other reports from the same authors (Elmoslemany *et al.*, 2009) that show a similar positive association between udder hygiene and bacteria in bulk tank milk, and the view that dirty udders and teats are an important source of enteric bacteria pathogen in milk (Pankey, 1989; Murphy & Boor, 2000; Galton *et al.*, 1986; Galton *et al.*, 1984). Other studies have also shown an association between reduced bacterial contamination of milk and the use of certain types of pre-milking teat dips and manual drying (Galton *et al.*, 1986; Magnusson *et al.*, 2006). Contaminated animal drinking water and poor management of dairy shed and other effluent can lead to increased pathogen cycling in dairy farms.

3.0 Materials of the Study

3.1 Sample Collection

The collection of the raw milk samples took place in Delta state (Agbor, Asaba & Warri) & Edo state (Auchi & Aduwawa) of Nigeria. A total of 30 raw milk samples (collected twice in different days) were analyzed. Samples were obtained from vendors and Fulani herdsmen. Sterile 25ml universal bottles with stoppers were used to collect the raw milk and packed in cooler containing ice, and transported from the point of collection to the laboratory for further analysis.

Figure: Sterilization Materials

3.2 Sterilization of Materials

3.2.1 Table and Benches: This was carried out by using a cotton wool soaked in 85% alcohol to swab the surface of both the table and benches in accordance to Cheesebrough (2000).

3.2.2 Glass wares: This was carried out using a hot air oven. That is, a double walled laboratory electric oven, made in China at 160 ^{O}C for 2h in accordance with report of Cheesebrough (2000).

3.3 Preparation and Sterilization of Media

All media were prepared according to manufacturer's specification. The media included nutrient agar and Mac conkey agar, and were autoclaved at 121^{0}c for 15 minutes.

3.3.1 Serial Dilution: This was carried out in accordance with the procedure reported by Cheesebrough (2004) as follows: Aliquot (1ml) of sample was serially transferred by means of sterile syringe into five test tubes containing 9ml of sterile water. 1ml each from test tubes (10^{-1} 10^{-2} 10^{-3} 10^{-4} 10^{-5}) were used to carry out pour plate technique according to procedure reported by Kanika (2009). The same procedure was carried out for the control.

3.3.2 Pour Plate Technique: This was carried out in accordance to the procedure reported by Kanika (2009) as follows:1ml of diluent from test tubes (10^{-1} 10^{-3} 10^{-5}) were aseptically transferred into sterile, empty labelled petri-dishes containing Nutrient agar and Mac conkey agar. The diluent and agar was mixed thoroughly by rotating the plates several times, clockwise, and then counter clockwise, in order to homogenize the raw milk sample and agars and to allow the organisms present in the raw milk to spread all over. The media was allowed to solidify; the plates were then inverted and incubated at 37^{o}C for 24hours for bacteria counts. After 24hours, the cultural characteristics of the colonies formed were noted and recorded.

3.4 Incubation

This was carried out in accordance to the procedure reported by Kanika (2009) as follows: All plates were incubated upside down to prevent drops of condensation on the inoculated surface. Plates were incubated using an incubator at 37^{o} C for 24h. At the end, the plates were examined for growth.

3.5 Sub Culturing

This was carried out in accordance to the procedure reported by Kanika (2009) as follows: after 24hours of incubation, colonies that grew on the nutrient agar and Mac conkey agar plate respectively were isolated and sub-cultured on sterile nutrient agar and incubated at 37^{o} C for 24h to obtain pure culture.

3.6 Identification of Isolates

3.6.1 Cultural Characteristics: This was carried out in accordance to the procedure reported by Cheesebrough (2004). The cultural characteristics of the various isolates were observed for features like shape, colour and nature of colonies.

3.6.2 Morphological Characteristics: This was carried out in accordance to the procedure reported by Cheesebrough (2004). A smear of test colony was made on grease – free slide with a loopful of sterile water, air dried and heat fixed. Thereafter the smear was flooded with crystal violet and allowed to stand for 55 secs, then the stain was poured off and it was placed at an angle downwards, Lugols iodine was applied to the smear and allowed to stand for 1 minute rinsed, the iodine was drained an washed with water The slide was again flooded with 95% ethanol for 30secs to decolorize the smear, after which, the alcohol was poured off and it was counter stained with safranin for 60 seconds before washing off with water. The slide was air-dried and observed under the microscope using oil immersion. Gram-positive bacteria appeared purple while gram negative bacteria appeared pink or red.

3.7Biochemical Tests

3.7.1 Aerobic Growth: This was carried out in accordance to the procedure reported by Buchanan and Gibbons (1994). The growth of the organism on the surface of the nutrient agar was recorded as aerobic growth.

3.7.2 Catalase Test: This was carried out in accordance to the procedure reported by Cheesebrough (2004). A smear of 24h-old culture test colony was made on clean grease – free slide. Using a sterile pipette, several drops of 3% hydrogen peroxide was added and observed for effervescence caused by liberation of oxygen gas. Catalase positive organisms produced active bubbles while catalase negative organisms did not.

3.7.3 Motility Test: This was carried out in accordance to the procedure reported by Cheesebrough (2004) as follows: Nutrient agar slant was prepared in test tubes as prescribed by the manufacturer, The needle was used to pick each test colony and stabbed at right angle and withdrawn immediately. The tubes were incubated for 24hours and observed for growth. A motile organism grew away from the line of stab while non – motile organism grew along the line of stab.

3.7.4 Oxidase Test: This was carried out in accordance to procedures reported by cheesbrough (2004) as follows: A small inoculums of culture plates were picked using sterile wire loop and place on whatman no. 1 filter paper. A drop of oxidase reagent was placed on the filter paper. Positive results include purple coloration within five to ten seconds of the analysis.

3.7.5 Citrate Test: Slants of the citrate medium were prepared in test tubes as prescribed by the manufacturer which is shown in appendix 1. Using a sterile wire loop, the organism was stabbed and incubated at 37^0c for 24 hours. The change in color from green to blue indicated positive result while negative is observed when it retained its green color or no change.

3.7.6 Indole Test: Glucose peptone phosphate broth was prepared according to the manufacturer's instruction, and was inoculated with the test organism using sterile inoculating wire loop and incubated at 37^0c for 48 hours.0.5ml of kovac's reagent was added to the broth culture and shaken gently and laid on the bench. A positive test is indicated by a red ring in the upper layer, while negative is observed when it retains it yellow color or no change.

3.7.7 Triple Sugar Iron Test: Triple sugar iron test agar were prepared and rest in a slant position, the bacteria isolates was stab on the bottom of the tube and was rub on the surface. It was incubated at 37^0c for 24 hours. The change in color at the bottom from light pink to yellow shows glucose is positive, change at the bottom and middle from light pink to yellow shows that glucose and lactose are positive. The presence of bubbles indicates gas production while change of color from light pink to black indicates hydrogen sulphide production (Cheesbrough, 2004).

3.8 Determination of Sensitivity Profile of Isolate

This was carried out in accordance with procedures reported by Cheesbrough (2004) as follows: Briefly commercially available antimicrobial disc (Oxoid ltd, Basingstoke, Hants, UK) of streptomycin (S= 10 µg/ml), Ciprofloxacin (Cip = 5 µg/ml), Ampicillin (A= 25 µg/ml), Rifampicin (R = 5µg/ml), Oxytetracyclin (OT= 30µg/ml) and chloramphenicol (C=30 µg/ml). Nutrient agar were prepared and dispensed into petri dish, allowed to solidify, an overnight broth culture which was compared with 0.5 Mcfarl and standard were swab on the surface of the medium and antibiotic disc was placed on the inoculated agar plates and incubated in the upright position overnight at 37^0c. The diameter around the disc which was expressed as the zone of inhibition were measured and recorded, all the organisms were susceptible to ciprofloxacin.

3.9 Total Bacterial Count (TBC)

Total viable count was carried out using the pour plate method described by Harrigan and MacCance (1976). Appropriate dilution (10^{-1} up to 10^{-6}) of the samples was plated on Nutrient agar and Mac conkeyAgar . The plates were incubated at 37^0C for 24 hours..

4.0 Research Methodology

The result obtained in this study showed the Prevalence of enteric pathogens in the raw milk samples obtained. The enteric pathogen isolated from the milk samples were *Pseudomonas sp., Proteus sp., Escherichia coli, Salmonella sp., enterobacter sp., Klebsiella sp*. as represented with their cultural and biochemical characteristics in table 4.1. In Table 4.2 to 5.1 shows the percentage occurrence of isolates in all five locations (Asaba, Agbor, Aduwawa, Auchi and Warri) with *E.coli* showing prevalence in all locations. Sensitivity test was also carried out on the organisms isolated, with all organisms being sensitive to Ciprofloxacin with varying zones of inhibition of 20mm, 16mm, 15mm, 17mm, and 16mm for *Proteus, Salmonella, E.coli, Pseudomonas sp.,Enterobacterrespectively*.

Table 4.1

Isolates	Cultural Characteristic	Morphological characteristics	Gram stain	Oxidase	Indole	Citrate	Catalase	glucose	H_2S	lactose	Motility
E.coli	Pink, ovoid with smooth edge	Rod	_	_	+	_	+	+	+	_	+
Enterobacter	Creamy,ovoid and discrete	Rod	_	_	_	+	+	+	_	+	_
Proteus sp.	Pink irregular and swarmy growth	Rod	_	_	_	_	+	+	+	+	+
P.aeruginosa	Bluish green and irregular growth	Rod	_	+	_	_	+	+	_	_	+
Salmonellla sp.	Creamy and irregular	Rod	_	_	_	_	+	+	+	_	_
Klebsiela sp.	Creamy ,mucoid and ovoid	Rod	_	_	_	+	+	+	_	_	_

Table 4.2 Prevalence of organisms in Aduwawa (Edo state)

Organism	No of sample collected	No of isolate (%)
E.coli	14	5 (35.7)
Proteus sp.	14	1 (7.1)
Klebsiella sp.	14	3 (21.4)
Pseudomonas sp.	14	2 (14.2)
Salmonella sp.	14	1 (7.1)
Enterobacter sp.	14	2 (14.2)

Table 4.3 Prevalence of organisms in Asaba (Delta state)

Organism	No of sample collected	No of isolate (%)
E.coli	14	6 (42.9)
Proteus sp.	14	1 (7.1)
Klebsiella sp.	14	3 (21.4)
Pseudomonas sp.	14	2 (14.2)
Salmonella sp.	14	1 (7.1)

Enterobacter sp.	14	1 (14.2)

Table 4.4 Prevalence of organisms in Auchi (Edo state)

Organism	No of sample collected	No of isolate (%)
E.coli	14	5 (35.7)
Proteus sp.	14	1 (7.1)
Klebsiella sp.	14	3 (21.4)
Pseudomonas sp.	14	2 (14.2)
Salmonella sp.	14	3 (21.4)

Table 4.5 Prevalence of organisms in Agbor (Delta state)

Organism	No of sample collected	No of isolate (%)
E.coli	14	4 (28.5)
Proteus sp.	14	2 (14.3)
Klebsiella sp.	14	3 (21.4)
Pseudomonas sp.	14	3 (21.4)
Salmonella sp.	14	2 (14.3)
Enterobacter sp.	14	1 (7.1)

Table 4.6 Prevalence of organisms in Warri (Delta state)

Organism	No of sample collected	No of isolate (%)
E.coli	16	5 (31.3)
Proteus sp.	16	2 (12.5)
Klebsiella sp.	16	4 (25.0)
Pseudomonas sp.	16	2 (12.5)
Salmonella sp.	16	2 (12.5)
Enterobacter sp.	16	1 (6.3)

Table 4.7 Prevalence of enteric pathogen in Aduwawa

Organism	No of sample collected	No of isolate (%)
E.coli	16	5 (31.2)
Klebsiella sp.	16	4 (25.0)
Pseudomonas sp.	16	3 (18.8)
Salmonella sp.	16	3 (18.8)
Enterobacter sp.	16	1 (6.3)

Table 4.8:Prevalence of enteric pathogen in Asaba

Organism	No of sample collected	No of isolate (%)
E.coli	17	5 (29.4)
Klebsiella sp.	17	4 (21.4)
Pseudomonas sp.	17	3 (17.6)
Salmonella sp.	17	3 (17.6)
Enterobacter sp.	17	2 (11.7)

Table 4.9: Prevalence of enteric pathogen inAuchi

Organism	No of sample collected	No of isolate (%)
E.coli	17	5 (29.4)
Proteus sp.	17	2 (11.8)
Klebsiella sp.	17	3 (17.6)
Pseudomonas sp.	17	3 (17.6)
Salmonella sp.	17	1 (5.9)
Enterobacter sp.	17	2 (11.8)

Table 5.0: Prevalence of enteric pathogen in Agbor

Organism	No of sample collected	No of isolate (%)
E.coli	14	5 (35.7)

Proteus sp.	14	1 (7.1)
Klebsiella sp.	14	3 (21.4)
Pseudomonas sp.	14	2 (14.2)
Salmonella sp.	14	1 (7.1)
Enterobacter sp.	14	2 (14.2)

Table 5.1: Prevalence of enteric pathogen in Warri

Organism	No of sample collected	No of isolate (%)
E.coli	18	5 (27.7)
Proteus sp.	18	3 (16.7)
Klebsiella sp.	18	4 (22.2)
Pseudomonas sp.	18	3 (16.7)
Salmonella sp.	18	2 (11.1)
Enterobacter sp.	18	1 (5.6)

Table 5.2: Zone of inhibition of antibiotics against enteric pathogen isolates

Organism	Ofx	Na	Pef	Cn	Au	Cpx	Sxt	S	Pn	Cep
E.coli	15	0	20	0	0	20	0	7	10	10
Pseudomonas sp.	18	0	10	16	0	20	15	0	20	0
Klebsiella sp.	20	0	0	0	17	23	20	0	0	20
Enterobacter sp.	0	0	20	22	0	24	19	0	24	0
Proteus sp.	25	0	15	20	0	19	21	21	20	0
Salmonella sp.	25	0	5	20	8	28	5	0	8	0

5.0 Discussions of the Results

The public health significance of this various organism isolated from raw milk is of great concern as it has been implicated in a number of food poisoning outbreaks. The isolation of this various organisms can be explained by their various distributions on plant, animal, in soil, on decomposed matter and as normal flora of skin and mouth. A total number of 30 raw milk samples were analyzed, from the result obtained; there were high contamination levels of Escherichia coli in raw milk, as it was prevalent in all locations. This is not surprising as Escherichia coli is an indicator organism of fecal origin. The presence of this enteric pathogen could be attributed to poor animal health (Sahin et al., 2012), poor milking practice (Blowey & Edmondson, 2010) or through other sources including, the skin of animal, infected dirty udder, the milker's hand, utensils and feces. Olatunji (2009) reported that the characterization of isolates through biochemical test revealed the presence of *Pseudomonas sp., Proteus sp., E.coli, Salmonella sp.,*

and Enterobacter sp. in the raw milk analyzed, the presence of this enteric pathogen in raw milk may be due to contamination from Water, soil, plant & the environment which is in agreement of the work done by Olatunji (2009). The antibiogram finding of the present study corresponds with those obtained by other researchers (Umar et al., 2006; Whong & Kwaga, 2007). In antibiotic sensitivity test, *E.coli* was resistant to nalidixic acid, augmentin and septrin, while Klebsiellawas resistant to nalidixic acid, gentamycin and refiacine, but was all sensitive to Ciprofloxacin as shown in table 5.2. Antibiotic resistance in microbes isolated from milk sample is still a major problem and being observed, the microorganisms found resistant are a threat to mankind.

6.0 Conclusions of the Study

This study found that the presence of these enteric pathogens in raw milk indicated contamination due to poor animal husbandry, unhygienic practices, inappropriate transportation, poor storage facilities & use of unsafe water. In addition, the authors reported in this study that the practice of drinking raw unpasteurized milk is hazardous because it increases risk of acquiring zoonotic diseases. The finding of this study provides insights into the enteric pathogen associated with consumption of raw milk. In order to ensure safety of raw milk, regulatory authorities should establish guidelines and standards based on research findings to cover the entire raw milk chain across Delta and Edo state.

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