ISOLATION, IDENTIFICATION, ASSESSMENT OF PATHOGENECITY AND ANTIBIOTIC SENSITIVITY OF *YERSINIA ENTEROCOLITICA* ISOLATES FROM PIGS

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Yersinia enterocolitica is an important zoonotic pathogen that causes yersiniosis in humans and animals. The present study was undertaken to detect *Y. enterocolitica* from pigs and foods of porcine origin by conventional cultural technique, which is not generally used owing to its cumbersome and time consuming nature. Isolation of *Yersinia* spp. by conventional microbiological techniques includes cold enrichment of samples for 3 weeks followed by alkaline treatment and selective isolation using specific media for *Yersinia* spp. like cefsulodin irgasan novobiocin (CIN) media along with *Yersinia* selective supplement. *In vitro* pathogenicity and antibiotic resistance pattern of the isolates recovered were also studied. Typical cultural and biochemical characteristics were used for the identification and confirmation of *Y. enterocolitica* isolates. Occurrence rate in pig faeces was 6.25 %; in pig tongues it was 7.5 %; in pig tonsils it was 15 % and it was 5 % in the ground pork samples. Occurrence rate was 8% with highest occurrence found in pig tonsils, whereas lowest in the ground pork samples. All the isolates were found positive for congo red binding test, crystal violet binding test and autoagglutination tests. *In vitro* sensitivity profile of the isolates against 12 commonly used antibiotics revealed that 100% of *Y. enterocolitica* isolates were sensitive to amikacin and gentamycin and resistant to erythromycin and ampicillin. Most of the isolates were sensitive for ciprofloxacin (87.5 %), norfloxacin (87.5 %), neomycin (87.5 %), tetracycline (75 %), nalidixic acid (75 %).

Key words: Antibiotic sensitivity, CIN agar, Foodborne infection, Pigs, *Yersinia enterocolitica*

Among the members of genus *Yersinia*, *Yersinia enterocolitica* is considered as the most important foodborne pathogen, and is recognized worldwide as an emerging food

borne zoonotic pathogen. *Y. enterocolitica* is a ubiquitous micro-organism indigenous to gastrointestinal tract of warm blooded animals like pig, dog, rodent etc. The

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organism has been isolated from a variety of foods like raw and pasteurized milk, unchlorinated drinking water, fresh vegetables, raw meat, shellfish, beef, oysters, mussels and environmental samples (Fredriksson-Ahomaa *et al.*, 2006; Subha *et al.*, 2009; Virtanen *et al.*, 2012; Paixao *et al.*, 2013). The significance of *Y. enterocolitica* is further strengthened by its capability to multiply at refrigeration temperatures and in vacuum packed food. Being reported from above 33 countries worldwide the incidence of *Y. enterocolitica* is apparently increasing (Bottone, 1999). Pigs are reported to be a major source of *Yersinia enterocolitica* and harbor the organism in their throat and tonsils (Funk *et al.*, 2000) as well as shed it in faeces (Rasmussen *et al.*, 1995). *Y. enterocolitica* has been frequently been isolated from slaughterline pigs (Bonardi *et al.*, 2003). Among numerous *Y. enterocolitica* serotypes, O:3, O:9, O:8, O:5 and 27 are associated with disease in humans and the main reservoir of serotype O:3 and O:9 is believed to be healthy pigs (Nesbakken *et al.*, 2003). The disease is characterized by enteritis leading to diarrhea, abdominal pain, cramps, fever and at times vomiting in young. In adults, pharyngitis, terminal ileitis and pseudo appendicitis syndrome are common. Secondary immunological complications may include erythema nodosum, reactive arthritis, Reiter's syndrome, glomerulo nephritis, myocarditis or thyroiditis (Cornelis *et al.*, 1987; Baumgartner *et al.*, 2007). In animals, enteritis and septicaemia is

commonly seen. However, many animals may remain as symptomless a carrier which is very important from public health point of view. Isolation of *Y. enterocolitica* is difficult, the current method for the isolation include enrichment followed by streaking on selective agar such as cefsulodin, irgasan and novobiocin medium (CIN) (Mehlmann *et al.*, 1978; Schiemann, 1979). Due to the cumbersome and time consuming isolation procedure, there are fewer reports of *Y. enterocolitica* from India. The study was undertaken with the objective to isolate and identify *Y. enterocolitica* from pigs and to test the isolates for their pathogenicity and antibiotic sensitivity.

MATERIALS AND METHODS

Reference bacterial strains: The bacterial strains used in this study were obtained from IMTECH, Chandigarh (*Yersinia enterocolitica* MTCC 840, MTCC 859 and MTCC 861). The strains were tested for their cultural, morphological, biochemical and pathogenic characteristics. All the strains were maintained by subculturing every month in brain heart infusion (BHI) slants.

Collection and processing of samples: A total of 300 samples from various sources (Pig farm IVRI, local private pig farms and slaughter house) were collected from different farms (Pig Farm IVRI, local private pig farms) and slaughter houses (IVRI slaughter house and local slaughter houses) in and around Bareilly, Uttar

Pradesh (Table 1). All the samples were collected in sterile conditions and transported to laboratory as quickly as possible for further processing.

Enrichment and Culturing: The fecal sample from pigs were inoculated into PBS containing 0.15% bile salts and 1% sorbitol and incubated at 4°C for 3 weeks. In case of tissue samples, about 10g of each tongue, tonsil and meat tissue of pig was cut into small pieces, and added in 90 mL PBS containing 0.15% bile salts and 1% sorbitol. The inoculated medium was then homogenized for 2 minutes in a stomacher. The homogenized medium was allowed to stand undisturbed at room temperature for 10 minutes to allow settling of large meat particles. Then the medium was incubated at 4°C for 3 weeks (Bercovier and Mollaret, 1984; ISO, 2003; Markey *et al.*, 2013). Following 3 weeks of cold enrichment in PBS containing 0.15% bile salts and 1% sorbitol, the inoculum was streaked onto cefsulodin irgasan novobiocin (CIN) plates, and incubated at 22°C for 7 days (Schiemann, 1979). Alternatively, 0.5 mL of enriched culture was mixed with 4.5 mL dilute aqueous alkali solution (0.5% KOH: 0.5% NaCl) for 15 sec and immediately streaked onto CIN agar (Delmas and Vidon, 1985; Fukushima, 1985; Luis *et al.*, 2013). The culture plates were checked daily for typical colonies for 7 days. Plating was also done on MacConkey agar and bismuth sulphite agar (BSA) (Cowan, 1975; Franzin *et al.*, 1984; Stern and Oblinger, 1980).

Identification of genus *Yersinia* and *Y. enterocolitica*: For confirmation of genus *Yersinia* Gram's staining, motility test and various biochemical reactions like oxidase test, urease test, citrate test, acid and alkali reaction on Kligle Iron agar (KIA) slants were performed (Cowan, 1975; Bercovier and Mollaret, 1984). The colonies of genus *Yersinia* were subjected to lysine, arginine and ornithine decarboxylase test, Voges-Proskauer (VP) test, methyl red test, phenylalanine (PA) deaminase test and motility for identification of *Y. enterocolitica* (Holt *et al.*, 1994; Markey *et al.*, 2013).

Bio-grouping of *Y. enterocolitica*: Positive cultures were subjected to nitrate reduction test, indole test, sugar utilization test, Esculin hydrolysis test, lipase test and deoxyribonuclease (DNase) test under modified Wauter's scheme for confirmation and bio-grouping of *Y. enterocolitica* (Bercovier *et al.*, 1980; Luis *et al.*, 2013).

***In vitro* pathogenicity testing and antibiotic sensitivity profiling of the isolates:** Isolates were subjected to auto-agglutination in MR-VP broth (Aulisio *et al.*, 1983; Markey *et al.*, 2013) and Congo red binding/ crystal violet binding assay (Prpic *et al.*, 1983; Bhaduri *et al.*, 1987; Luis *et al.*, 2013) for testing the virulence. The recovered isolates were tested against commonly used antibiotics (Table 2) using antibiotic discs (Hi Media) according to the criteria of the National Committee for Clinical Laboratory Standards by disc diffusion technique (Bauer *et al.*, 1966; NCCLS, 2002).

RESULTS

Isolation studies: Among total 200 samples subjected for the bacterial isolation, 16 were found positive for *Y. enterocolitica* and the sources of the isolation are shown in Table 1.

Cultural / colony characteristics: Typical dark red colonies surrounded by transparent

border; usually flat with smooth border and entire edge giving a characteristic “Bulls-Eye” appearance, were observed after 72 hrs of incubation on CIN agar (Fig. 1A) in case of positive samples, whereas in MacConkey agar, the colonies were pale pink (Fig. 1B) and in bismuth sulphite agar they were black enamel like (Fig. 1C). Gram staining revealed gram negative coccobacillary morphology.

Table 1. Details of the samples collected and subjected for bacterial isolation

Sl. No.	Sample type	Sample No.	Sample positive (%) for <i>Y. enterocolitica</i>
1	Pig faeces	80	5 (6.25%)
2	Pig tongues	40	3 (7.5%)
3	Pig tonsils	40	6 (15%)
4	Pork	40	2 (5%)
Total number of samples		200	16 (8%)

Table 2. Antibigram of *Y. enterocolitica* isolates (n=16)

Sl. No.	Antibiotic (Conc.)		Antibiogram of isolates n (%)		
			Sensitive	Intermediate	Resistant
1	Streptomycin	(10 mcg)	10 (62.5%)	3 (18.75%)	3 (18.75%)
2	Nalidixic acid	(30 mcg)	12 (75%)	2 (12.5%)	2 (12.5%)
3	Gentamycin	(10 mcg)	16 (100%)	0 (0%)	0 (%)
4	Tetracycline	(30 mcg)	12 (75%)	0 (0%)	4 (25%)
5	Amikacin	(10 mcg)	16 (100%)	0 (0%)	0 (%)
6	Erythromycin	(15 mcg)	0 (0%)	0 (%)	16 (100%)
7	Norfloxacin	(10 mcg)	14 (87.5%)	2 (12.5%)	0 (%)
8	Ampicillin	(10 mcg)	0 (0%)	0 (%)	16 (100%)
9	Neomycin	(30 mcg)	14 (87.5%)	0 (%)	2 (12.5%)
10	Cephalothin	(30 mcg)	0 (%)	2 (12.5%)	14 (87.5%)
11	Ciprofloxacin	(10 mcg)	14 (87.5%)	2 (12.5%)	0 (%)
12	Chloramphenicol	(30 mcg)	10 (62.5%)	3 (18.75%)	3 (18.75%)

Biochemical analysis and biotyping:

Colonies which were citrate negative (-), urease positive (+), and gave an alkaline slant/acid butt without gas or H₂S on KIA were used for species identification and biogrouping (Table 3). The isolates recovered under the present study were positive for catalase, glucose, maltose, mannose, sucrose, xylose, maltose, D-xylose, sorbitol, manitol, whereas rhamnose, indole, raffinose, ornithine, oxidase, citrate utilization test, lysine, arginine, they showed negative results. Esculin hydrolysis and lipase yielded variable results. On the basis of motility at 25°C, negative citrate utilization test, positive nitrate reduction test and positive Voges-Proskauer test, the isolates were confirmed as *Y. enterocolitica* (Table 3). Most of the isolates belonged to biotype 1A and few belonged to biotype 3.

In vitro pathogenicity testing: In MR-VP broth, the tube incubated at lower temperature (25°C) showed turbidity, whereas the tube incubated at 37°C showed positive agglutination of bacteria along the bottom/wall of test tube and clear supernatant fluid. Positive isolates displayed tiny red colonies and small intensely purple colonies in congo red binding test and crystal violet binding test, respectively. All the isolates were positive for congo red binding test, crystal violet binding test and autoagglutination tests.

Antibiotic sensitivity profile of *Y. enterocolitica* isolates: The isolates recovered under present study showed high sensitivity to gentamycin, amikacin, ciprofloxacin, neomycin, norfloxacin and tetracycline, whereas moderate sensitivity

Table 3. Biochemical properties of *Y. enterocolitica*

Name of the biochemical test	Standard / Reference isolate	Test / Study isolate	Name of the biochemical test	Standard / Reference isolate	Test / Study isolate
Catalase test	+	+	Lysine decarboxylase	-	-
Oxidase test	-	-	Ornithine decarboxylase	+	+
Indole test	+	+	Sucrose fermentation	+	+
MR test	+	+	Rhamnose fermentation	-	-
VP test	+	+	Raffinose fermentation	-	-
Citrate utilization	-	-	Xylose fermentation	+	+
Glucose fermentation	+	+	Maltose fermentation	+	+
Sorbitol fermentation	+	+	Mannose fermentation	+	+
Urease test	+	+	Lipase test	+/-	+/-
Arginine decarboxylase	-	-	Esculin hydrolysis	+/-	+/-
Trehalose fermentation	+	+	Nitrate reduction	+	+
KIA	+/-	+/-	Deoxyribonuclease	-	-

towards streptomycin and chloramphenicol (Table 2). The isolates were resistant against ampicillin, cephalothin and erythromycin (Table 2).

DISCUSSION

Yersinia enterocolitica, formerly referred to as *Bacterium enterocoliticum*, *Pasterulla pseudotuberculosis* type B and *Pasteurella* X, is a ubiquitous, gram-negative bacterium pathogenic to human beings (Schleifstein and Coleman, 1943; Frederiksen, 1964). *Y. enterocolitica* is a psychophilic food and water borne entero-invasive pathogen causing enterocolitis, acute mesenteric lymphadenitis, mimicking appendicitis, post infectious arthritis, systemic infections, nausea, vomiting, diarrhoea, septicemia, skin and eye infection in humans (Baumgartner *et al.*, 2007). Pigs are regarded as a major reservoir and asymptomatic carrier of *Y. enterocolitica*. In the present study, various aspects related to microbiological isolation (cultural and biochemical characterization of the isolates) of this fastidious pathogen was explored to assess the chances of food borne infection to human.

Under the present study, among 200 samples 16 were found positive for *Y. enterocolitica*. Pig tonsils (15%) and pig tongue (7.5%) showed maximum occurrence of infection followed by pig faeces (6.25%) and ground pork samples (5%). Likewise, earlier investigators have reported higher rate of prevalence of *Y. enterocolitica* in pig tonsils and tongue, while low incidence was reported in ground pork (De Boer and Nouws, 1991;

Fredriksson-Ahomaa *et al.*, 2009; Arora, 2010; Baghel and Kumar, 2017). Arora (2010) in his study also reported higher occurrence of *Y. enterocolitica* in pig tonsils (34.74%) as compared to ground pork (4.35%). The variations in the occurrence may be attributed to difference in methods of sample collection, enrichment broths, enrichment methods and incubation steps used. Pigs are regarded as a major reservoir of *Y. enterocolitica* and asymptotically carry the pathogen particularly in their tonsils and tongue. Therefore, the consumption of porcine tongue and tonsils in particular is considered a risk factor (Bottone, 1999). In case of asymptomatic carriers, pathogens from these infected tissues may contaminate the carcass during slaughter and dressing procedure (Nesbakken *et al.*, 2003).

Isolation of *Y. enterocolitica* from food is more difficult than from clinical specimens with active infections because the pathogen may be present in low numbers, and so requires enrichment in liquid media prior to streaking on solid media (Schiemann, 1982). Nowak *et al.* (2006) reported that the use of PBS with 1% sorbitol and 0.15% bile salts for enrichment of samples for 2-3 weeks followed by streaking on CIN agar was the most sensitive cultural method for detection of *Y. enterocolitica* in pig fecal contents and the second most sensitive method for detection of *Y. enterocolitica* in pig tonsils. Although the method was time consuming but yielded satisfactory result under the present study. *Y. enterocolitica* appeared as 0.5 to 1.0 mm

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A

B

C

**Fig. 1. (A) Colonies of *Y. enterocolitica* on CIN agar
(B) MacConkey agar Bismuth
(C) Sulphite agar plates**

colonies with a red “Bull’s-Eye” and a clear border (Fig. 1A) as reported previously (Schiemann, 1979). Dark enamel like colonies (Fig. 1C) on bismuth sulphite agar were characteristic as reported by other workers (Stern and Oblinger, 1980). Although, CIN agar has been reported to be inhibitory to most of the food borne pathogens, but, some strains of *Citrobacter* spp. and *Enterobacter* spp. imitates similar colonial morphology, hence to distinguish *Y. enterocolitica* from non-yersiniae bacteria, a battery of biochemical tests were employed. The results of the biochemical tests (Table 3) confirmed the presence of *Y. enterocolitica* in the samples which were in agreement with the findings of Bercovier and Mollaret, 1984; Holt *et al.*, 1994.

Among 200 samples, 16 isolates of *Y. enterocolitica* were recovered by cultural method. For biotyping of *Y. enterocolitica*, modified Wauter’s scheme was adopted (Bercovier *et al.*, 1980; Bottone, 1999). Most of the isolates belonged to biotype 1A and few belonged to biotype 3 (Table 3). Clinical, epidemiological and experimental evidence suggests that few biotype 1A strains of *Y. enterocolitica* are virulent, and can cause gastrointestinal disease (Tennant *et al.*, 2003) and have been implicated in nosocomial (Ratnam *et al.*, 1982) and food-borne (Greenwood and Hooper, 1990; Butt *et al.*, 1991) outbreaks of yersiniosis. Biotype 1A strains produce heat stable enterotoxin which invades cultured epithelial cells (Grant *et al.*, 1998), and

resist killing by macrophages (Grant *et al.*, 1999). The biotype 1A strains (serotype O: 6, 30) have also been reported to cause placentitis and abortion in pregnant ewes (Corbel *et al.*, 1992).

In vitro pathogenicity testing included autoagglutination testing, congo red binding and crystal violet binding. All the isolates were positive for congo red binding test, crystal violet binding test and autoagglutination tests. The results of biotyping and *in vitro* pathogenicity testing were comparable to that reported by other workers in India (Singh and Viridi, 1999; Singh *et al.*, 2003).

All the strains of *Y. enterocolitica* isolates from different samples were sensitive to amikacin and gentamycin (100 %). Most of the strains were sensitive for ciprofloxacin (87.5 %), norfloxacin (87.5 %), neomycin (87.5 %), tetracycline (75 %) and nalidixic acid (75 %). *Yersinia enterocolitica* isolates were highly resistant to erythromycin (100%), ampicillin (100%) and cephalothin (90%). Susceptibility of the isolates to amikacin and gentamycin and resistance to ampicillin, erythromycin and cephalothin were in accordance with earlier studies (Singh and Viridi, 2004; Kushal and Anand, 2001; Baumgartner *et al.*, 2007; Subha *et al.*, 2009).

The present study revealed that *Y. enterocolitica* are present in human food of porcine origin and therefore due precaution must be undertaken during handling of pork and raw pig tissues. Microbiological methods, although

cumbersome and time consuming may be used for isolation and identification of the pathogen from environmental (including food) samples. Presence of biotype 1A of *Y. enterocolitica* raises a concern as it is capable of producing GI disease in human. However, more studies are required on this particular pathogen from different areas of the country for getting a complete picture of the prevalence and risk factor associated

with this food borne pathogen.

Conflict of interest: Authors declare that there is no conflict of interest regarding the present research work.

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