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Molecular Characterization of Diarrhoegenic *Escherichia coli* Isolated from Neonatal Goat-Kids

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ABSTRACT

Faecal samples (n=300) from diarrhoeic neonatal goat-kids of different livestock sheds of ICAR-CIRG, Makhdoom, and field goat-kids of Mathura, UP were aseptically collected, and used for *E. coli* isolation. On the basis of cultural, morphological, biochemical and molecular characteristics, a total of 193 *E. coli* isolates were identified from 300 fecal samples. Out of 140 *E. coli* isolates, only 90 isolates could be serotyped at National *Salmonella* and *Escherichia* Centre, Central Research Institute, Kasauli, and the most common serogroups responsible for neonatal diarrhoea were found as O88 (n=11), O22 (n=10), O11 (n=8) and O83 (n=7). Congo red dye agar test was done to determine invasiveness of the isolates, and 77.20% (149/193)*E. coli* isolates showed Congo red binding activity. Identification of shiga toxin producing *E. coli* (STEC) was done by PCR amplification of its *stx-1* gene, and 5.69% (11/193) isolates were identified as STEC. Pathotype specific primers were used to amplify bundle forming pilus (*bfpA*) gene of enteropathogenic *E. coli* (EPEC), and 35.23% (68/193) isolates were identified as EPEC. A multiplex PCR was performed to detect labile toxin producing enterotoxigenic *E. coli* (ETEC-lt), stable toxin producing enterotoxigenic *E. coli* (ETEC-st) and enteroinvasive *E. coli* (EIEC), and 24.35% (47/193), 2.59% (5/193) and 2.07% (4/193) isolates were determined as ETEC-st, ETEC-lt and EIEC, respectively. EPEC and ETEC-st were found as the most prevalent pathotypes associated with neonatal diarrhoea in goat-kids whereas; O88 and O22 were observed as the most common serogroups in causing diarrhoea in the neonatal goat-kids.

Keywords: *E. coli,* neonatal diarrhoea, goat-kids, pathotypes, PCR.

Diarrhea is documented as a frequent cause of neonatal mortality among animals including goats throughout the world (Sherman, 1987). The mortality due to diarrhea may go as high as 60%, and *E. coli* scours (colibacillosis) is the single major cause of death in neonatal goat kids (Kritas, 2002). Colibacillosis is frequently seen in kids less than 10 days of age, but is the most common at 1to 4 days of age (Radostits *et al.,*1999). *Escherichia coli* (*E. coli*), the causative agent of colibacillosis, is generally a normal commensal gram-negative rod-shaped bacterium that lives inside the intestinal tracts of humans and warm blooded animals. Based on the type of virulence factor present and host clinical symptoms, pathogenic *E. coli* strains associated with diarrhoea are categorized into six pathotypes: (i) enteropathogenic *E. coli* (EPEC) (ii) enterotoxigenic *E. coli* (ETEC) (iii) shiga toxin producing *E. coli* (STEC) orverotoxin producing *E. coli* (VTEC) (iv) entero-aggregative *E. coli* (EAEC) (v) entero-invasive *E. coli* (EIEC) (vi) diffusely adherent *E. coli* (DAEC) (Nataro and Kaper, 1998).

STEC produce toxins similar to the one secreted by *Shigella dysenteriae*. That is why, the said E. *coli* are known as STEC. Pathogenic potential of shiga toxin can be determined by vero cell toxicity assay (Beutin *et al*., 2007). Hence, STEC are also called verotoxin or vero-cytotoxin-producing *E. coli* (VTEC). STEC can cause

diarrhoea in human and animals, but most of time, animals act as source of infection for the human beings (Nataro and Kaper, 1998). STEC causes hemorrhagic colitis in humans, which may progress into hemolytic uremic syndrome. The STEC causing hemorrhagic colitis is also called entero-hemorrhagic *E. coli* (EHEC). VTEC strains produce shiga toxins 1 and/or 2 (*stx*1, *stx*2) while EHEC produce *stx*1 and/or *stx*2 and intimin (*eae*A). Likewise, the ETEC strains produce heat labile (lt) and/or heat stable (st) enterotoxins, while EPEC strains may possess EPEC adherence plasmid (EAF) that carries the bundle forming pilus (*bfp*A) gene. The EIEC strains have the ability to invade the colon epithelial cells due to presence of a plasmid that carries the *ial* gene. The EAEC strains are characterized by presence of aggregative adherence factors. Molecular methods are the most suitable techniques for differentiating diarrhoegenic strains of *E. coli* from nonpathogenic ones (Nataro and Kaper, 1998). The aim of this work was to identify pathogenic *E. coli* strains isolated from neonatal goat kids affected with diarrhoea.

MATERIAL AND METHODS

Sample collection

During April 26, 2016 to December 31, 2017; more than 1000 neonatal goat kids from different livestock sheds of the Institute (ICAR-CIRG, Makhdoom, Mathura), and of field goats of various parts of district Mathura (Uttar Pradesh), were examined for presence of diarrhoea. The study area was situated in the semi-arid zone of western Uttar Pradesh of India (27.10º- 27.50º N, 77-78.0º E, MSL-169.2). The temperature of the area varies from 28 ºC to 49 ºC in summer (April to July) and 1 to 24 ºC in winter (November- February). The kidding season in the area mostly falls during February to May and August to October. Among the population studied, the targeted neonatal kids showed diarrhoea with clinical signs including weakness, dehydration and soiled perineal region. Fecal samples were collected from diarrhoeic neonatal goat kids (n=300) using sterile swabs (Himedia). The collected swabs were kept on the ice, and immediately brought to the Laboratory, Division of Animal Health, ICAR-Central Institute for Research on Goats, Makhdoom, Farah, Mathura (UP), India.

Isolation and identification of *E. coli*

All fecal samples were inoculated on sterile blood agar (5% de-fibrinated sheep blood) and incubated at 37ºC for 18-24 hr. Then, colonies from the blood agar were inoculated on MacConkey's agar, and kept overnight at 37ºC. The pink colored colonies (showing lactose fermentation) grown on MacConkey's agar, were further re-inoculated on Eosin Methylene Blue (EMB) agar, and incubated at 37ºC for 18-24 hr.

Biochemical tests

For biochemical identification of *E. coli*, the tests such as Catalase, Oxidase and IMViC were performed as per the standard bacteriological protocols (Cowan and Steel, 1975).

Detection of pathogenicity of *E. coli*

Congo red dye agar test was carried out to determine the invasiveness (pathogenicity) of the *E coli* isolatesas per the technique recommended by Berkhoff and Vinal (1986). The test was performed using soyabean casein digest medium containing 0.03% Congo red dye. Appearance of red colonies within 72 hr was recorded as a positive reaction (invasive *E. coli*). The colonies which did not bind the dye, and remained white or grey even after 72 hr, and were declared negative for Congo red dye binding activity.

Serotyping of isolates

A total of 140 isolates of *E. coli* were sent to National *Salmonella* and *Escherichia* Centre, Central Research Institute, Kasauli, India for the serotyping.

Molecular characterization of *E. coli*

The confirmatory identification of *E. coli* was done by PCR amplification of the universal stress protein A (*usp*A) gene using species specific primers are presented in Table 1 as used by Mishra *et al.* (2017). Thereafter, the identification of shiga toxin producing *E. coli (*STEC) or verotoxin producing *E. coli* (VTEC) was done by PCR amplification of its *stx-1* gene as per the protocol of Islam *et al.* (2007) (Table 1). Likewise, self designed pathotype specific

Table 1: Details of the PCRs, target gene, primer used and annealing temperature in the Current Study

Sl. No.	Specificity of the PCR for	Target Gene	Primers	Annealing Temperature (°C) / Time (min)
1	*E. coli*	*usp*A	F-5'-CCGATACGCTGCCAATCAGT-3'	55/0.5
			R-5-ACGCAGACCGTAGGCCAGAT-3'	
2	STEC	*stx-1*	F-5'-CACAATCAGGCGTCGCCAGCGCACTTGCT-3'	58/0.5
			R-5'-TGTTGCAGGGATCAGTCGTACGGGGATGC-3'	
3	EPEC	*bfp*A	F-5'- ATGGTGCTTGCGCTTGCTGC-3'	57/0.5
			R-5'-AATCCACTATAACTGGTCTGC-3'	
4	ETEC-lt	*lt*	F-5'-GGC GAC AGA TTA TAC CGT GC-3'	50/0.75
			R-5'-CGG TCT CTA TAT TCC CTG TT-3'	
5	ETEC-st	*st*	F-5'- ATT TTT CTT TCT GTA TTG TCT T-3'	50/0.75
			R-5'-CAC CCG GTA CAA GCA GGA TT-3'	
6	EIEC	*ial*	F-5'- GGTATGATGATGATGAGTCCA-3'	50/0.75
			R-5'-GGAGGCCAACAATTATTTCC-3'	

primers (F-5'- ATGGTGCTTGCGCTTGCTGC-3' and R-5'-AATCCACTATAACTGGTCTGC-3'was used to amplify the bundle forming pilus A (*bfp*A) gene of enteropathogenic *E. coli* (EPEC). (Table 1). A multiplex PCR was performed to identify labile toxin producing enterotoxigenic *E. coli* (ETEC-lt) and stable toxin producing enterotoxigenic *E. coli* (ETEC-st), and enteroinvasive *E. coli* (EIEC). The protocol of Lopez *et al.* (2003) was used in the above PCR.

RESULTS AND DISCUSSION

Diarrheic samples were inoculated on the sterile blood agar plates, and incubated at 37° C for 24 hr. The growth on blood agar showing catalase positive, oxidase negative and Gram negative reactions (Gram negative rods) was then inoculated on MacConkey agar, and incubated at 37° C for 24 hr. Pink colored colonies (Fig. 1) on MacConkey agar were further inoculated on Eosin Methylene Blue agar (EMB), and incubated at 37ºC for 24 hr. Colonies with a characteristic metallic green sheen on EMB agar (Fig. 2) were indicative of E. *coli.* The results of IMViC tests were obtained as +ve, +ve, –ve, -ve.

E. coli has six *usp* genes namely A, C, D, E, F and G, and the *usp*A gene is important for survival of *E. coli* during cellular growth, adhesion and motility (Nachin *et al.,* 2005). Synthesis of *usp* protein is induced in response to stresses such as heat shock, nutritional starvation, osmotic pressure and the presence of toxic agents etc. (Nyström and Neidhardt, 1992).

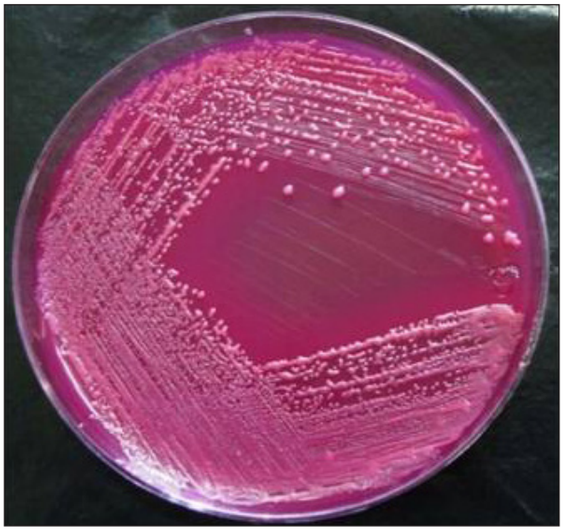

Fig. 1: *E. coli* as pink colored colonies showing lactose fermentation on MacConkey agar

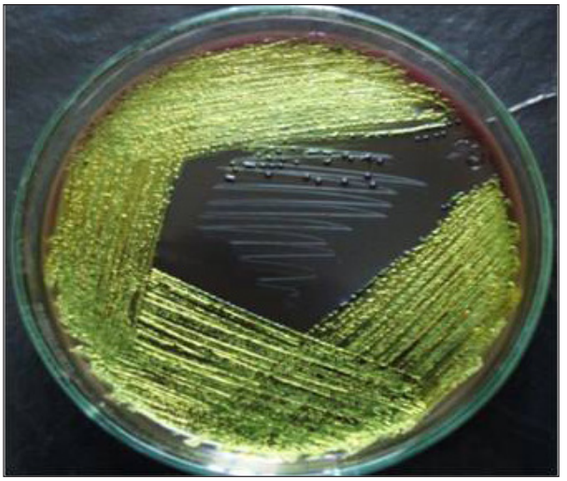

Fig. 2: *E. coli* showing characteristic green metallic sheen on EMB agar

The molecular identification of *E. coli* was done by PCR amplification of the universal stress protein A (*usp*A) and the size of the amplified product was found 884 bp (Fig. 3).

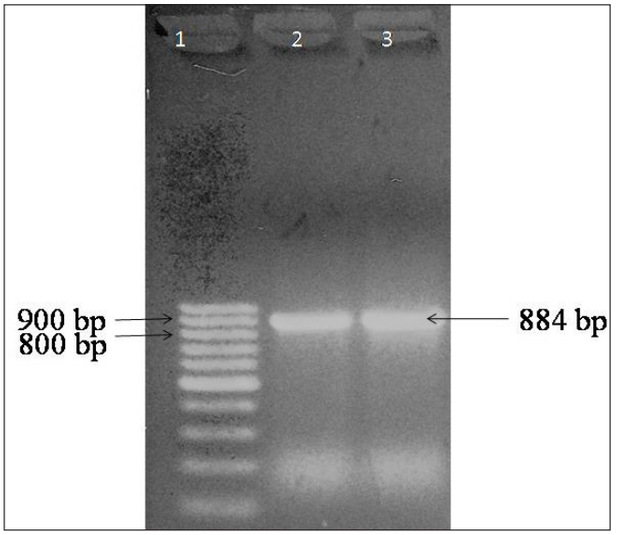

Fig. 3: PCR amplification of *usp*A gene of *E. coli.* Lane 1: Molecular weight marker; Lane 2 & 3: Amplified *usp*A gene

From 300 fecal samples, 193 isolates of *E. coli* were identified on the basis of cultural, morphological, biochemical and molecular characteristics (Table 1). The increase in *usp*A synthesis appears to be the result of induction of the monocistronic *uspA* gene (Nystrom and Neidhardt, 1992; Nystrom and Neidhardt, 1994). Hence, *usp*A gene was used for molecular identification of *E. coli*, and amplified product was obtained as 884 bp. similar finding was also reported by Chen and Griffiths (1998).

E. coli are enteroinvasive pathogen which has the ability to penetrate and multiply within intestinal epithelial cells. Virulence of these bacteria is associated with multiple genes which are located on plasmid and chromosome. These genes encode various functions which include attachment to host cells, induction of endocytosis, intracellular multiplication, and spread to adjacent cells (Stugard *et al*., 1989). Congo red binding activity distinguishes between invasive and non-invasive *E. coli* (Chambers *et al*., 1985; Daskaleros and Payne, 1986). In the current study, Out of 193 *E. coli* isolates, 77.20 % (149/193) isolates showed Congo red binding activity (Table 2; Fig. 4), that was, invasive ones whereas; Nashwa *et al.* (2010), in his study, reported that all the tested isolates were invasive in nature (100 % Congo red binding activity).

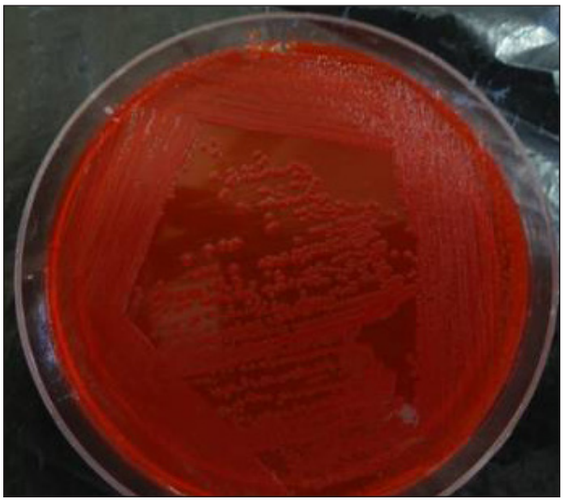

Fig. 4: *E. coli* as brick red colonies showing Congo red binding activity

The lipopolysaccharide on the surface of *E. coli* constitutes the 'O' antigens which are the important virulence factors, and play important role in host-pathogen interaction. The 'O' antigens are responsible for antigenic specificity of the strain, and determine the 'O' serogroup, important for classification of *E. coli* strains for epidemiological studies, in tracing the source of outbreaks of gastrointestinal or

Table 2: Details of the Identified *E. coli* Isolated from Neonatal Goat-Kids Affected with Diarrhoea

Breeds of goat	Number of fecal samples collected	Number of *E. coli* isolates obtained	Invasive *E. coli* showing positive Congo Red dye activity	STEC**	EPEC**	ETEC-st**	ETEC-lt**	EIEC**
Jamunapari	118	80	64	02	21	20	2	1
Barbari	117	59	45	03	25	15	—	2
Jakhrana	15	14	12	02	07	4	—	—
Multiple*	14	13	09	03	06	3	—	—
Local (Field)	36	27	19	01	09	5	3	1
Total	**300**	**193**	**149**	**11**	**68**	**47**	**5**	**4**

* Different breeds (Jamunapari, Barbari, Jakhrana & Sirohi) maintained at the Experimental Sheds, ICAR-CIRG, Makhdoom, Farah-281122, Mathura; **Shiga Toxin Producing *E. coli* (STEC), Enteropathogenic *E. coli* (EPEC), Stable Toxin Producing Enterotoxigenic *E. coli* (ETEC-st), Labile Toxin Producing Enterotoxigenic *E. coli* (ETEC-lt) and Entero-invasive *E. coli* (EIEC).

other illness, and for linking the source to the infection. (Deb Roy *et al*., 2011).

In present study, a total of 140 isolates of *E. coli* were sent to National *Salmonella* and *Escherichia* Centre, Central Research Institute (CRI), Kasauli for the serotyping. Out of 140 *E. coli* isolates, only 90 isolates could be serotyped by CRI. Among 90 isolates, the most common serogroups responsible for neonatal diarrhoea were found as O88 (n=11), O22 (n=10), O11 (n=8) and O83 (n=7) (Table 3) whereas, Pachaury and Kataria (2012) found O55 as the most frequently isolated serogroup from diarrheic lamb and goat kids. In our study, the percentage of un-typable isolates were observed as 4.5% (5/90) whereas, in the study carried out by Nashwa *et al.* (2010), the percentage was reported quite higher, that is 26.67%.

STEC are the important cause of diarrhea throughout the world (Bielaszewska and Karch, 2000). Shiga toxin (stx) is believed to be the cardinal virulence factor of STEC (O'brien *et al*., 1992). The prevalence of STEC among the *E. coli* isolates in the present study was found as 5.69% (11/193) by PCR amplification of *stx-1* gene (Fig. 5). Wani *et al*. (2003) reported similar results with 6% prevalence rate. However, Bhat *et al*. (2008) and Türkyılmaz *et al*. (2014) reported somewhat higher rate (17.8%) than that of our finding. Further, in another finding, the rate has been reported up to 32% (Bandyopadhyay *et al*., 2011).

The prevalence of EPEC among *E. coli* isolates of present study was 35.23% (68/193) (Fig. 5), and it was reported lower (20.5%) in the study conducted by Singh *et al*. (2018), but higher (68.07%) by Türkyılmaz *et al*. (2014).

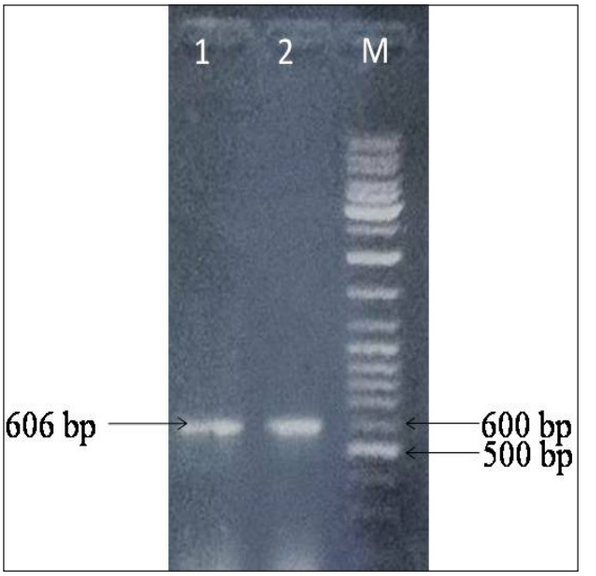

Fig. 5: PCR amplification of *stx-1* gene of *VTEC.* Lane M: Molecular weight marker; Lane 1& 2: Amplified PCR products of *stx*-1 gene

Likewise, out of 193 *E. coli* isolates; 24.35% (47/193), 2.59% (5/193) and 2.07% (4/193) isolates were identified as ETEC-st, ETEC-lt and EIEC, respectively (Fig. 6, 7 & 8). ETEC were found as the second most common pathotype (after EPEC) associated with neonatal diarrhoea in goat-kids whereas, Kritas (2002), in his study, reported that ETEC was the principal pathotype responsible for neonatal diarrhoea. While in the other study, the main pathotype associated with diarrhea in kids was EPEC (Cid *et al.,* 1996).

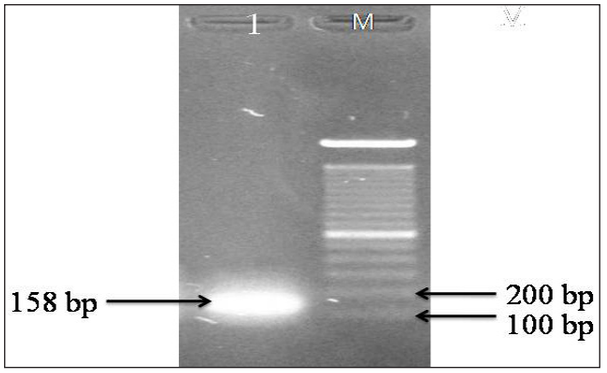

Fig. 6: PCR amplification of *bfp*A gene of EPEC. Lane M: Marker; Lane 1: Amplified gene product

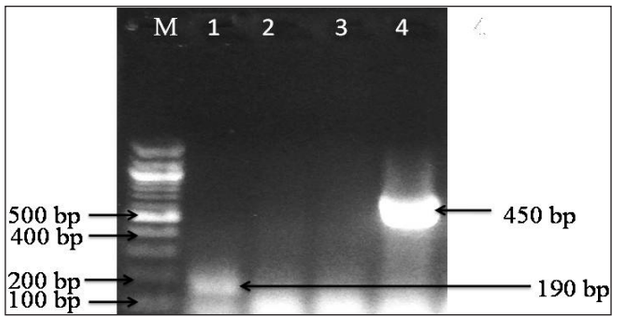

Fig. 7: Multiplex PCR For Identification of ETEC. Lane M: Molecular Marker; Lane 1 : Amplified PCR Product (ETEC-st); Lane 2 & 3: Negative; Lane 4: Amplified PCR Product (ETEC-lt)

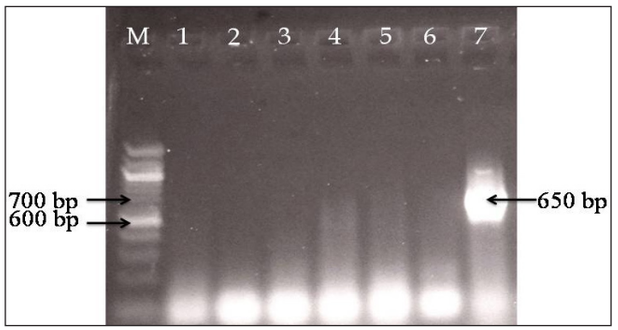

Fig. 8: Multiplex PCR For Identification of EIEC. Lane M: Molecular Marker; Lane 1-6: Negative; Lane 7: Amplified PCR Product

On the basis of above findings, it may be concluded that different serogroups and pathotypes of *E. coli* were responsible for diarrhoea in neonatal goat-kids. EPEC and ETEC were found as the predominant pathotypes associated with neonatal diarrhoea in goat-kids. Likewise, O88 and O22 of *E. coli* were observed as the most common serogroups in causing diarrhoea in neonatal goat-kids.

Table 3: Serogroups of the Identified *E. coli* and their frequency

E. coli **Isolate Number**	**Serogroup**	**Frequency**
1	O11	10
2	O11	10
3	O4	1
4	O5	1
5	O10	1
6	O11	10
7	-ve	44
8	O9	1
9	O11	10
10	O141	3
11	-ve	44
12	O11	10
13	O49	2
14	-ve	44
15	-ve	44
16	O11	10
17	O22	10
18	O49	2
19	O35	3
20	-ve	44
21	-ve	44
22	-ve	44
23	O141	3
24	-ve	44
25	O22	10
26	O22	10
27	O88	12
28	O7	5
29	O118	4
30	O118	4
31	O35	3
32	-ve	44
33	O20	2
34	-ve	44
35	O118	4

36	O149	1	81	-ve	44
37	-ve	44	82	O11	10
38	-ve	44	83	O22	10
39	-ve	44	84	-ve	44
40	-ve	44	85	O8	5
41	O118	4	86	O11	10
42	O115	1	87	O34	4
43	O22	10	88	-ve	44
44	O20	2	89	-ve	44
45	O126	2	90	O22	10
46	-ve	44	91	-ve	44
47	-ve	44	92	-ve	44
48	O141	3	93	-ve	44
49	O35	3	94	-ve	44
50	O88	12	95	-ve	44
51	O63	2	96	O2	2
52	O34	4	97	-ve	44
53	O34	4	98	-ve	44
54	O34	4	99	-ve	44
55	O8	5	100	-ve	44
56	O128	11	101	-ve	44
57	O157	1	102	-ve	44
58	O1	1	103	O22	10
59	-ve	44	104	O22	10
60	O88	12	105	O83	7
61	-ve	44	106	-ve	44
62	O17	1	107	O83	7
63	O88	12	108	Un-Typed (UT)	4
64	O11	10	109	UT	4
65	O22	10	110	O11	10
66	O16	1	111	O88	12
67	-ve	44	112	O88	12
68	O114	1	113	O145	2
69	O126	2	114	Rough (R)	2
70	O88	12	115	O88	12
71	-ve	44	116	O2	2
72	-ve	44	117	O7	5
73	-ve	44	118	O88	12
74	-ve	44	119	O8	5
75	-ve	44	120	O8	5
76	-ve	44	121	O88	12
77	O63	2	122	UT	4
78	O22	10	123	O26	1
79	O7	5	124	O145	2
80	-ve	44	125	-ve	44

126	-ve	44
127	R	2
128	O8	5
129	O88	12
130	O83	7
131	O83	7
132	O7	5
133	O88	12
134	O83	7
135	O7	5
136	O83	7
137	O101	1
138	O83	7
139	UT	4
140	UT	4

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